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REVIEW ARTICLE

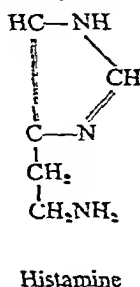
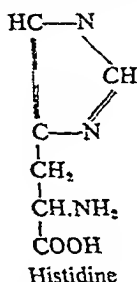
THE ANTIHISTAMINE DRUGS

By D. M. DUNLOP

B.A.(Oxon.), M.D., F.R.C.P.(Edin.), F.R.C.P.

*Professor of Therapeutics and Clinical Medicine in the University of Edinburgh,
Chairman of the British Pharmacopœia Commission*

Histamine is a base formed from the amino-acid histidine by the removal of the carboxyl group from the latter substance, which change can be brought about by some bacteria or by prolonged heating with acids. It is present in most cells in the body, but it only becomes



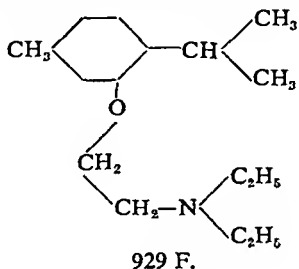
active when liberated from the cell by trauma or in some other way. It is normally present in large quantities in the intestine from which it is absorbed to some extent, and is destroyed by the enzyme histaminase which is present in particularly high concentration in the intestinal mucosa.

The pharmacological actions of histamine vary according to the experimental animal into which it is injected. Contraction of plain muscle occurs which may be followed by an inhibitory phase. This constricting effect has a striking species specificity. It is mainly to be observed in the bronchioles of the guinea-pig, the muscular tissue round the hepatic veins of the dog and in the pulmonary arteries of the rabbit. Capillary dilatation also takes place, causing a fall in blood pressure and shock, which is particularly obvious in the cat. This action can be demonstrated on the human skin by scratching it through a drop of histamine solution or by liberating histamine from the cells by moderate trauma as may be caused by drawing a blunt pointed instrument firmly across the skin. The characteristic triple response may then be seen: redness, due to dilated capillaries; a weal, due to exudation of plasma from the capillaries under the epidermis; a flare, due to an axon reflex. A higher concentration of histamine will cause itching in addition to the weal formation. Lastly, histamine is a strong stimulant of gastric secretion. This action is used as a clinical test of gastric function and is not antagonised by atropine.

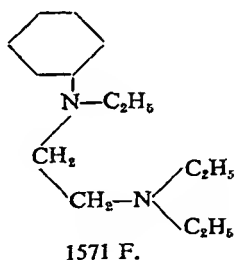
Chemical methods of detecting histamine and of estimating it quantitatively are extremely laborious and difficult, and most of our know-

azoprotein. There may still be a future for work along these lines, but up to the present the benefits of such treatment have not been striking and no-one who has had considerable experience of the use of histamine azoprotein, which may produce some clinical benefit, can think it is the philosopher's therapeutic stone in dealing with allergic disorders.

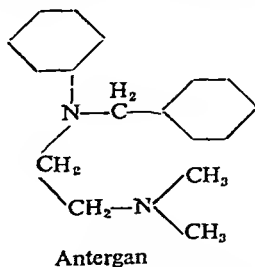
From 1933 onwards French workers had been systematically searching for synthetic antihistamine drugs, and between 1937 and 1939 certain active compounds were actually discovered which would protect guinea-pigs against anaphylactic shock and lethal doses of histamine, but which were, however, too toxic for human use. The first of these substances, thymoxy-ethyl-diethylamine was discovered by Staub and Bovet and



labelled 929F. in their series, and the second discovered by Staub was another Fournau compound containing an ethylenediamine radical labelled 1571F.



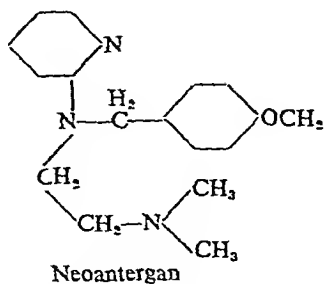
In 1942 antergan (2339 R.P.)—a phenyl-benzyl-dimethyl-ethylene-diamine compound—was introduced by Halpern, and soon the results of



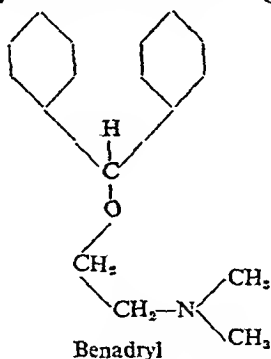
its experimental and clinical trial began to appear in the French literature. Such was the chaotic state of Europe at that time, however, that Hal-

THE ANTIHISTAMINE DRUGS

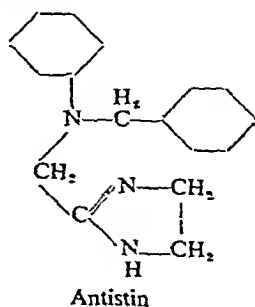
pern's discoveries did not become generally known till after the liberation of France, by which time neoantergan (2786 R.P.) had also been introduced.



It differed from antergan in the replacement of a benzene by a pyridine group and the addition of a methoxy group to the benzene ring, and was a more potent, specific and less toxic antihistamine. In 1945 pyribenzamine and benadryl were introduced in America, in which country both drugs began to be widely used therapeutically, though for some time benadryl was the only antihistamine to be generally employed in



Britain. Pyribenzamine differs from neoantergan in the absence of a methoxy group on the benzene ring, and benadryl is dimethylaminoethylbenzhydryl ether hydrochloride.



In attempts to produce more potent and less toxic antihistamine drugs new derivatives of these compounds have been studied recently. Antistin is closely related to antergan, but the dimethylamino linkage is replaced

by an imidazole ring, while hetramine is the pyrimidine analogue of pyribenzamine. Two further compounds— α -naphthyl-methylethyl- β -chloroethylamine and β -2-biphenyloxyethyl- β -chloroethylamine are halogen derivatives of neoantergan. They are of interest because they antagonise the action of adrenaline, whereas all the earlier antihistamines, with the exception of the original one, 929F., potentiate the action of adrenaline. The search for more potent and less toxic antihistamines continues, and it may well be that we have as yet only touched the fringe of new developments.

Potency. The potency of antihistamine substances has been tested in a number of ways. The lethal dose of histamine injected intravenously is determined for a group of guinea-pigs, which usually varies from 0.4 to 0.8 mg./kg. of body weight. The antihistamine to be tested is then injected subcutaneously and increasing doses of histamine are thereafter given to determine the maximum dose which the animal survives, and therefore the protective effect of the antihistamine. The second test is designed to discover the protective action of the antihistamine against the lethal effect of histamine inhaled by a guinea-pig. The third test determines the power of the antihistamine to prevent the action of histamine in causing contraction of the guinea-pig's isolated intestine. The fourth test determines its effect in preventing the depressor action of histamine on a dog's blood pressure, and the fifth test measures its power to abolish or diminish the size of the weal caused by an intradermal injection of histamine.

The relative antihistamine activity of the drugs which have been commonly employed in clinical practice has been tested by these animal experiments. Using such tests it was found that benadryl and the old-fashioned French preparation, antergan, were less effective than pyribenzamine, and all of them very much less effective than neoantergan. The relative clinical effectiveness of the various drugs is, however, not nearly so divergent in human beings as the experiments on laboratory animals would have led us to expect, though it does seem that neoantergan is at present the most potent and specific antihistamine which we possess.

Mode of Action. A knowledge of the mode of action of this group of drugs is necessary if they are to be used efficiently. It is apparent that they might act in a number of ways: they might prevent the release of histamine from the tissues; they might abolish its action by entering into some inert chemical combination with it; they might set up a directly antagonistic pharmacological action; or they might block the action of histamine by competing with it successfully for the tissue receptors. If they acted by preventing the formation of histamine they would have no effect on the production of the typical skin weal when histamine is injected subcutaneously, but they do have a striking effect in this respect. Further they would prevent the stimulating action of histamine on the gastric secretion, which, as we shall see, does not occur. There is no evidence whatever that they destroy histamine or render it inactive by

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entering into chemical combination with it. The only type of pharmacological antagonistic action to histamine which is at all likely would be the potentiation of adrenaline, but as some potent antihistamines antagonise rather than potentiate adrenaline it is impossible to believe that there can be any relationship between the sympathomimetic and antihistamine properties of the others. Thus, by a process of *reductio ad absurdum* we come to the conclusion that antihistamines act by blocking the action of histamine by combining with its tissue receptors.

If this theory is correct it is apparent that the underlying allergic or anaphylactic tendency persists in spite of the use of antihistamines since the abnormal production of histamine is not interfered with, and, therefore, the administration of the antihistamine in clinical practice has to be continued either indefinitely in a few cases or at least till the allergic or anaphylactic tendency has subsided spontaneously, or as the result of artificial or natural desensitisation. It is thus important to realise that the use of these drugs does not absolve the physician from considering the advisability of specific desensitisation in certain cases, though such desensitisation may be frequently impossible, undesirable or unnecessary.

Other Effects. Few drugs have only one property. Most of them produce—perhaps to a lesser degree—effects additional to that for which they are principally prescribed in therapeutics. Antihistamines are no exception to this rule, for, besides abolishing the effects of histamine, they have many other actions, some of which are inconvenient when they are used in clinical practice. In addition to their antihistamine activity, they have to a varying extent anti-acetylcholine, local anæsthetic and sympathomimetic or sympatholytic properties. Antispasmodic, analgesic, and quinidine-like actions have also been demonstrated by some members of this group. Benadryl has, even in therapeutic doses, a pronounced atropine-like action, causing dryness of the mouth and some dilatation of the pupil, an analgesic action causing drowsiness and some slight spasmolytic effect. These properties are shared, but to a less extent, by neoantergan and pyribenzamine. As we have seen, however, some of the newer antihistamines and 929F. are sympatholytic drugs. Antihistamines are local anæsthetics. Neoantergan, benadryl and antistin, for instance, are 3·3, 2·5 and 1·5 times as potent as procaine. When taken by the mouth, however, they do not produce a demonstrable local anæsthetic effect on the skin. Their power as antihistamines has nothing to do with their local anæsthetic effect, since the latter wears off in about an hour's time, whereas their antihistamine action lasts for at least four hours. Neoantergan has been found to be twice as powerful as quinidine on the auricle of the rabbit, but this effect has not been demonstrated in the human subject. Thus, as Burn has pointed out, antihistamines "join the group of other substances which include spasmolytics like trasentin and syntropan, analgesics like pethidine and papaverine, local anæsthetics like procaine, and atropine-like substances. None of these can be sharply distinguished from one another. Probably each possesses

every property in some degree." The common properties of all these drugs suggest that their site of action must be a similar one.

Dosage and Administration. Antihistamine drugs are usually given by the mouth in tablets or capsules. Benadryl and pyribenzamine are prescribed in doses of 50 to 100 mg., with a maximum daily dose in the case of benadryl of 400 mg. and in the case of pyribenzamine of 600 mg. Neoantergan and antistin, being less toxic, can be given in bigger doses of 100 to 200 mg. with a maximum daily dose of 800 mg. Children tolerate the drugs well, and over the age of twelve can be given the adult dose, with appropriately smaller doses under that age. The drugs are quickly absorbed and fairly quickly excreted in the urine. The effect of a single dose does not last for more than six hours, so that in order to maintain a satisfactory concentration, dosage should be well spaced, the drug being given at least three times a day. In severe cases four doses should be administered—the last one as late as possible at night so as to "cover" the hours of sleep. The tablets or cachets should be swallowed whole and not chewed as they have an unpleasant taste, and if brought in contact with the mucous membrane of the mouth or pharynx will have a marked local anæsthetic effect. Neoantergan and antistin are not spasmolytics like benadryl, and their use may, indeed, produce increased motility of the alimentary tract in experimental animals. In consequence they may occasionally cause nausea if given on an empty stomach, and should, therefore, be taken after food. Tolerance to antihistamines does not seem to take place.

Benadryl is procurable in a purified solution containing 10 mg. of the drug per ml. for intravenous use and its administration in this way has been recommended for anaphylactic emergencies, but otherwise the parenteral use of antihistamines is unnecessary and may, indeed, cause on occasion rather alarming symptoms of collapse. As might be expected from the mode of action of antihistamines, no effect, apart from diminishing skin irritation, is produced on established lesions, which will subside spontaneously, though new lesions are prevented from occurring. Thus antihistamines, even if given intravenously, will have little effect on an established allergic emergency such as swelling of the tongue or œdema glottidis, for which adrenaline is the drug of choice.

Antihistamines may be used in a suitable base for local application in some of the itching dermatoses, and may be applied locally to the nose in cases of allergic rhinorrhœa. For this latter purpose antistin is the most suitable preparation.

Side Effects.—No deaths or toxic effects leading to organic change have occurred as the result of the administration of antihistamines, even though they have been administered to some patients for years. Side effects are, however, very common, and in about 5 per cent. of cases may be sufficiently distressing to necessitate discontinuing their use. A full dose of benadryl or antistin will cause effects in about 50 per cent. of cases. Pyribenzamine and neoantergan are less toxic, but cause side

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effects in about 30 per cent. of cases. The practical superiority of neo-antergan, and to a less extent pyribenzamine, over benadryl lies in the fact that they are not only more active antihistamines milligramme for milligramme, but can be tolerated in larger doses and may thus benefit some patients for whom the necessarily smaller dose of benadryl had proved ineffective. The great majority of the side effects of antihistamines only occur when the drugs are first administered and tend to wear off in a few days.

The principal side effect of all antihistamines, but particularly benadryl, is sleepiness, fatigue or dizziness. In some patients on first taking the drug this hypnotic effect may be very marked indeed, and may in a few cases persist even after they have been taking the drug for a long time. Patients should be warned about this effect before they are given antihistamines, and, until their reaction to the drug in this respect has been ascertained, should not take their first few doses before undertaking work requiring skilled judgment. It is wise also to start treatment with a small daily dose and gradually to increase it till the optimum effect has been obtained, as in this way the patient usually becomes quickly tolerant to any hypnotic effect which may be present. Alternatively, 5 mg. of amphetamine may be administered coincidently in the morning and at mid-day for the first few days of treatment till the hypnotic effect of the antihistamine wears off. Owing to the fact that antihistamines often produce soporific effects when they are first taken, the coincident use of hypnotics and sedatives should be prescribed with care. On the other hand, a curious sensation of tension, nervousness and unreality is occasionally produced by antihistamines, and these sensations may lead to insomnia rather than to sleepiness.

As benadryl has a strong atropine-like action, it is not surprising that patients frequently complain of dryness of the mouth as the result of its use. Pyribenzamine, neoantergan and antistin may also produce this side effect, but less commonly and to a less extent. Atropine and its congeners should not, therefore, be prescribed along with these drugs, though there is no contra-indication to the coincident use of sympathomimetic preparations.

Other side effects have occasionally been noted as the result of the therapeutic use of antihistamines, but they are rare and unimportant.

Therapeutic Uses. It might be anticipated that drugs which antagonise histamine would have a wide range of therapeutic application. Their value in treatment, however, is actually somewhat limited to allergic conditions characterised by vascular reactions in the skin and mucous membranes resembling the effects produced by the local application of histamine. Thus they may be claimed almost as specifics in cases of acute and chronic urticaria or angioneurotic oedema, and in many of the urticarias encountered when a patient becomes sensitive to drugs such as penicillin, liver extract and insulin. The itching of pruritus

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vulvæ and ani is often greatly ameliorated by the use of antihistamines, while they either cure or very much alleviate some 75 per cent. of cases of hay fever and a somewhat smaller proportion of cases of perennial vasomotor rhinitis.

The administration of histamine does not cause bronchospasm in healthy persons, but it has this effect in asthmatic subjects, just as it has in guinea-pigs, and this artificially produced bronchospasm can be prevented by antihistamine drugs. These observations do not *prove* that naturally occurring asthma is due to a simple release of histamine or that it can be prevented by antihistamine drugs, but they encouraged the hope that such drugs might be of some value in the prevention and treatment of the condition. The results of their clinical trial in asthma are, however, extremely conflicting, and much of the work claiming antihistamines to be of value in the disorder is based on poorly controlled evidence. It is at any rate certain that the benefits to be derived from the use of antihistamines in asthma, if they exist, are in no way comparable to their value in the allergic reactions in the skin and mucous membranes mentioned above.

It is almost certain that histamine is the natural stimulant of gastric secretion. It might be expected, therefore, that antihistamine drugs would be of value in the treatment of hyperchlorhydric dyspepsia and peptic ulcer. All clinical and experimental evidence unfortunately goes to show that they are of no practical use in these conditions and have no significant effect in modifying gastric secretion. The drugs have also been tried in a great variety of other allergic states with negative results.

In summary, then, antihistamine drugs are of great value in superficial allergies, in the treatment and prevention of which they constitute a major therapeutic advance, but they are of little or no value in the treatment of the more deep-seated visceral allergies in the human subject. It may be that histamine does not play a part, or a predominant part, in the production of some of these visceral disorders and that this may account for the failure of antihistamines to influence their course favourably. We do know, however, that histamine does stimulate gastric secretion, and that in spite of this antihistamine drugs have no influence on this action of histamine. It may be, therefore, that in some visceral allergies histamine is released in such intimate contact with the effector cell that antihistamines are impotent to block its action.

RESEARCH PAPERS

HYDROLYTIC CHANGES IN SOLUTIONS OF STILBAMIDINE

By J. D. FULTON and T. W. GOODWIN

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HENRY¹, working in the Sudan, suggested that solutions of 4:4' diamidinostilbene dihydrochloride (stilbamidine dihydrochloride), underwent a number of changes, including hydrolysis of the amidine groups to the corresponding mono- and di-amides, when kept in diffuse daylight. He² isolated a substance shown by analysis to be 4-carbamyl-4'-amidinostilbene hydrochloride. From kinetic considerations he deduced that a dimer of stilbamidine was also formed. The same author³ gave further experimental details when this investigation had been completed. Fulton⁴ found by chemical methods that, in this country, 1:2:3:4-tetra-(4'-amidinophenyl)-cyclobutane was the only product formed on exposure of stilbamidine solutions to light. On account of our interest in this subject Drs. Henry and Kirk in 1947 kindly sent us some bottles of the old solutions of stilbamidine dihydrochloride prepared in the Sudan in 1941 and 1942, in which we have confirmed the presence of 4-carbamyl-4'-amidinostilbene and have also obtained 4:4'-dicarbamylstilbene in pure form. The same substances have now been obtained by us from stilbamidine solutions kept at 37°C. for long periods or on autoclaving the solutions for a few hours. Oastler and Fidler⁵ described cerebral lesions in dogs following intravenous administration of stilbamidine solutions which had been autoclaved for a short period. Sen Gupta⁶ suggested that the drug had been affected by this treatment. From toxicity experiments with mice we have not obtained any evidence in support of the latter view.

EXPERIMENTAL

Six bottles of 1 per cent. solution of stilbamidine dihydrochloride of approximately 100 ml. volume were received by us from the Sudan in September, 1947, with the information that they had been prepared in 1941-42 and kept in the dark or in diffused daylight. The contents (see Table I) were yellowish in colour and large crystals as well as some micro-crystalline material were present, the latter being sometimes very adherent to glass. The two substances were readily separated by their different solubilities in water, in which the larger crystals dissolved. The soluble material after several recrystallisations were shown by analysis to have the composition of 4-carbamyl-4'-amidinostilbene monohydrochloride with two molecules of water of crystallisation. (Found: in solid dried at 90°C.; C, 63.33; H, 5.30; N, 13.85, 14.1; Cl, 21.1; loss at 90°C. 10.45, 10.64 per cent. $C_{16}H_{16}ON_2Cl$, requires C, 63.66; H, 5.34; N, 13.93; Cl, 11.76; H_2O . in hydrated material 10.66 per cent.) The substance

crystallised in thin laths from water, in which it is less soluble than stilbamidine dihydrochloride and had no m.pt. up to 320°C. Its aqueous solution contained Cl⁻ ions and rapidly decolorised aqueous bromine or

TABLE I
PRODUCTS PRESENT IN OLD SOLUTIONS OF STILBAMIDINE FROM THE SUDAN

Number of sample					pH of solution	Total solid	Water-insoluble portion
1	6.8	mg. 590	mg. 200
2	6.4	505	57
3	6.2	460	30
4	6.4	525	33
5	6.2	395	40
6	6.4	180	37
TOTAL					...	2655	397

permanganate solutions. When mixed with ammonium nitrate in excess a yellowish somewhat insoluble nitrate was formed which crystallised in fine rods from water m.pt. around 290°C. as given by Henry². The aqueous solutions of 4-carbamyl-4'-aminostilbene hydrochloride and those of stilbamidine dihydrochloride showed a similar blue fluorescence and their absorption spectra were identical. The microcrystalline material, which proved to be the diamide, was insoluble in common organic solvents but soluble to a limited extent in acetic acid and was somewhat more soluble in ethylene glycol, from which plates and fine rods were respectively obtained with no m.pt. up to 320°C. (Found: N, 10.7 per cent. $C_{16}H_{14}O_2N_2$ requires N, 10.53 per cent.)

The solutions fluoresced blue except number 6, which had a greenish tinge. Spectrophotometric analysis showed that samples 1 and 2 contained only saturated material, formed from the parent substance by the action of light, while sample 3 contained only the original material. In samples 4, 5 and 6 both substances were present. Deposits had never been observed by us in this country from solutions of stilbamidine dihydrochloride kept for more than a year at laboratory temperatures, which did not exceed 20°C., in light or dark. A series of solutions was therefore subjected, in stoppered bottles, to different conditions of light and temperature and observed over a period of 6 months; the initial pH of the solutions was approximately 6.8 and did not alter appreciably during the experiment. The results obtained are shown in Table II.

The product was practically all monoamide, with only negligible traces of diamide, and formed crystals in some cases 2 cm. long. Temperature is apparently of importance in the reaction as no amides were formed at that of the laboratory. It also seems as if light exerted some influence since solutions kept in complete darkness yielded relatively very small amounts of amide. Good yields of both amides were obtained by autoclaving stilbamidine dihydrochloride solutions; this proved a rapid and convenient method of obtaining both products as shown in Table III.

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It appears from the data recorded that hydrolysis of the amidino groups in stilbamidine is readily accomplished by heating solutions of the dihydrochloride under pressure. The amides are much less soluble

TABLE II

RESULTS OF KEEPING STILBAMIDINE SOLUTIONS AT 37° C. FOR A PERIOD OF 6 MONTHS

Number of sample	Solutions of dihydrochloride	Conditions of keeping	First appearance of deposit	Percentage yield
1 2	100 ml. of 1 per cent. 50 ml. of 1 "	At 37° C. exposed to electric light for short intervals	8 weeks 6 weeks	31 22
3 4 5 6	100 ml. of 1 " 100 ml. of 1 " 100 ml. of 0.2 " 100 ml. of 1 "	Complete darkness at 37° C.	— 16 weeks 17 weeks 8 weeks	None 5 2 2.5
7 8	100 ml. of 1 " 100 ml. of 1 "	Complete darkness at room temperature 5° to 20° C.	— —	None None

than the parent substance and the yields recorded in the table represent the solid obtained on cooling the treated solutions. Because of the much greater solubility of the parent di-isethionate and resulting monoamide salt it is more satisfactory to start with the dihydrochloride. When a

TABLE III

RESULTS OF AUTOCLAVING SOLUTIONS OF STILBAMIDINE UNDER DIFFERENT CONDITIONS

100 ml. of solution		Treatment in autoclave		Percentage yield	
Nature	Strength	Atmospheres	Hours	Monoamide	Diamide
4 : 4' diaminodistilbene dihydrochloride	1.0 per cent.	1	1	None	None
	1.0 "	1	1	Trace	—
	1.0 "	1½	4	40	7
	0.5 "	1½	4	24	5
	1.0 "	1½	7	52	16
	1.0 "	2	4	45	11
4 : 4' diaminodistilbene di-isethionate	1.0 "	1½	4	7	Trace
	0.5 "	1½	4	10	Trace
	10.0 "	1½	2	2.5	Trace
4-carbamyl-4'-aminodistilbene ...	1.0 "	1½	2	—	Trace

solution of the monoamide was treated under the above conditions, conversion to the corresponding diamide took place only to a slight extent. It was found by analysis and spectrophotometric measurements that the mono- and di-amides prepared by us in different ways are identical with the products formed in the Sudan. The absorption spectra of these two substances are indistinguishable from that of the parent *trans*-stilbamidine. The values obtained for the latter and the mono-

amide in aqueous solution and of the diamide in acetic acid were as follows:

	λ max	ϵ max
4:4'-diamidinostilbene dihydrochloride	328 m μ	37,800
4-carbamyl-4'-amidinostilbene hydrochloride ...	328 m μ	38,200
4:4'-dicarbamylstilbene	328 m μ	40,000 (approx.)

The value ϵ for the diamide is only approximate on account of its extreme insolubility. The fact that its spectrum was observed in acetic acid does not invalidate comparison with the monoamide since the latter's spectrum was unchanged in this solvent. The two groups $>C=O$ and $>C=NH$ thus appear to be chromophorically identical.

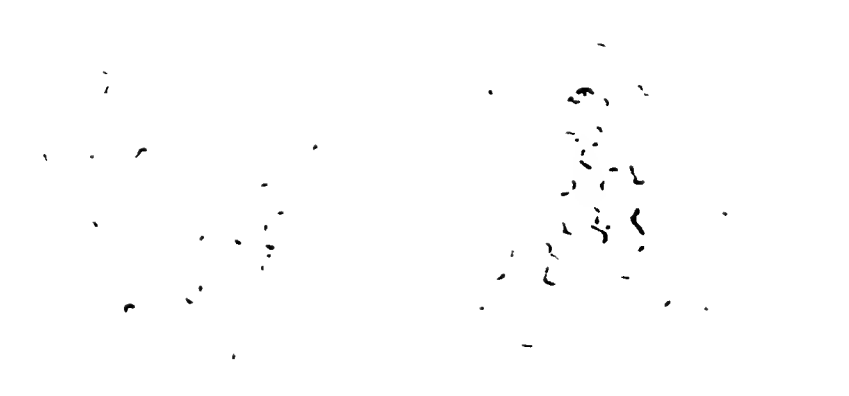
Various methods have been reported for the estimation of stilbamidine, for example fluorescence (Henry and Grindley⁷); colour reaction with glyoxal (Devine⁸), (Fuller⁹); spectrophotometric (Fulton and Goodwin¹⁰); fluorophotometric (Saltzman¹¹). None of these methods is entirely satisfactory. Wien¹² and Hampton¹³, using the fluorimetric and colorimetric methods to estimate excretion of stilbamidine in the urine of laboratory animals, found that the values obtained by the latter method were much higher than those obtained by measurement of fluorescence. In view of these results and the possession of similar optical properties by the monoamide and parent substance it seems unlikely that the former is a metabolic product of stilbamidine. Their toxicities as well as those of solutions of stilbamidine autoclaved at 5 lb. pressure for 20 minutes, as used by Oastler and Fidler⁵, have been compared in mice. The results are shown in Table IV.

TABLE IV

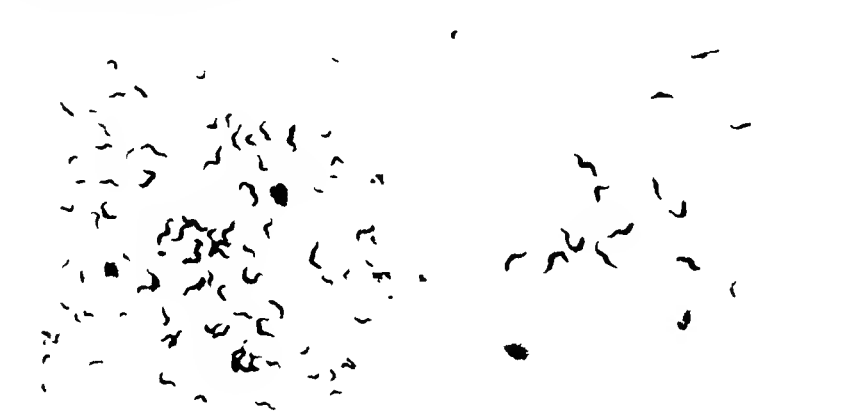
TOXICITY OF VARIOUS SUBSTANCES FOR MICE
 D—Died in less than 1 hour after injection
 P—Died within a few days of injection
 S—Survived observation period of 1 week

Drug	Nature of solution	Effect of doses (mg./20 g. mouse intraperitoneally)		
		2.0	1.0	0.5
4:4' diamidinostilbene dihydrochloride	Fresh	6D 2P 2S / 10	2P 18S / 20	5S/5
	Autoclaved at 5 lb. pressure for 20 minutes	5D/5	1P 15S / 16	5S/5
	Autoclaved at atmospheric pressure for 20 minutes	5P/5	2P 18S / 20	5S/5
4-carbamyl-4'-amidino-stilbene	Fresh	6D/6	9D 3S / 12	6S/6

They indicate that 4-carbamyl-4'-amidinostilbene is more toxic than stilbamidine for mice and also that autoclaving of the latter solutions even



Figs. 1 and 4. Appearance under ultra-violet illumination of *T. congolense* and *T. rhodesiense* respectively. Exposed *in vivo* to 4:4'-diamidinostilbene.



Figs. 2 and 5. The same parasites respectively exposed under the same conditions to 4'-carbamy1-4-amidinostilbene.

lense and *T. rhodesiense* respectively not exposed to drug.

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at a pressure of 1 atmosphere has not led to significant increase in toxicity.

Therapeutic tests on mice infected with *T. rhodesiense* and *T. congolense* were carried out with 4-carbamyl-4'-amidinostilbene hydrochloride as shown in Table V. It was not possible to test 4:4' di-carbamylstilbene in the same way on account of its insolubility.

TABLE V
RESULTS OF TREATMENT OF TRYPANOSOME INFECTED MICE.
R Blood free from trypanosomes, but relapse occurred.
N Blood never free from trypanosomes.

Mice infected with :	Drug	Effect of doses (mg./20 g. mouse intraperitoneally)	
		0.5	0.25
<i>T. rhodesiense</i>	4-carbamyl-4'-amidino stilbene hydrochloride	1R/15N/16	6N/6
<i>T. congolense</i>		2R/14N/16	1R/5N/6

It is evident that 4-carbamyl-4'-amidinostilbene is inactive therapeutically in the above infections and the life of each mouse was prolonged for only a short period. On the other hand 4:4'-diamidinostilbene is curative at high dilutions in the former infection, but requires a dose approaching the maximum tolerated (1 mg./20 g. mouse) to eradicate *T. congolense* infections. In order to find out the distribution of these drugs in trypanosomes exposed *in vivo* to their action, use was made of their similar fluorescent properties. For this purpose mice heavily infected with *T. rhodesiense* and *T. congolense* were treated with a solution of 0.5 mg. of each drug intraperitoneally and 1 hour later when the trypanosomes were still actively motile, blood smears of treated and untreated animals were made on a quartz slide. The slide was mounted dry without cover glass on the Beck-Barnard ultra-violet microscope and the object was illuminated by means of a quartz dark ground illuminator using the group of lines of the magnesium spark spectrum at 2830Å. Micrographs were taken using a Zeiss 4 mm. apochromat (N.A. 0.95) and a Zeiss No. 2 projection ocular. The length of exposure was 2 minutes in each case. The correction collar on the objective was adjusted to give the best image at a magnification of X 150. The appearances produced are those shown in Figures 1 to 6.

In the case of both trypanosomes exposed to stilbamidine selective absorption of the drug has occurred, as shown by the presence in them of two bright granules. The position of one granule corresponds to that of the blepharoplast, but the nature of the other at the anterior end has not been determined. The remainder of the cytoplasm does not fluoresce more brightly than that of untreated trypanosomes. There is absence of fluorescence in accompanying red cells which are not readily visible. The

inactive 4-carbamyl-4'-amidinostilbene appears to have been generally absorbed throughout the bodies of the trypanosomes as indicated by the increased brightness compared with that of parasites not exposed to the drug.

SUMMARY AND CONCLUSIONS

The formation of 4-carbamyl-4'-amidinostilbene and 4:4'-dicarbamylstilbene from solutions of stilbamidine had been shown to occur when the latter were maintained for a number of weeks at 37°C., in diffuse light, and to a lesser extent when kept at the same temperature completely in the dark. When the same solutions were maintained at temperatures which varied from 5° to 20°C. the formation of amides did not take place. Henry's observations made in the Sudan have been confirmed. Good yields of the amides were obtained by autoclaving solutions of the parent substance at 1 to 2 atmospheres pressure for several hours. The monoamide was inactive against *T. rhodesiense* or *T. congolense* infections of mice and does not appear to be selectively absorbed by the trypanosomes like the active stilbamidine. The fact that solutions of stilbamidine autoclaved under the conditions employed by Oastler and Fidler undergo no demonstrable change and are not more toxic for mice than similar solutions freshly prepared, suggests that the lesions encountered by these authors in dogs were due to unchanged stilbamidine.

Grateful acknowledgment is made to Mr. J. Smiles and Mr. F. V. Welch, of the National Institute for Medical Research, for the micrographs.

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THE INHIBITION OF ADRENALINE BY SPASMOLYTIC AGENTS

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IT HAS LONG BEEN KNOWN that¹ certain fibres in the autonomic nervous system which are classified on anatomical grounds as post-ganglionic sympathetic fibres are functionally cholinergic in nature. The effects of stimulation of such fibres are not reproduced by injection of adrenaline, but are potentiated by previous administration of eserine and are abolished by atropine sulphate. Bussel² has collected much evidence, from a review of earlier literature on the subject and from new experimentation, which supports the view that atropine has an inhibitory action upon the effects of stimulating post-ganglionic adrenergic sympathetic fibres and upon the actions of adrenaline itself. These investigations were carried out upon various preparations of portions of the vascular bed of animals and upon the contraction of the nictitating membrane in cats. It was found that in contrast to the behaviour of atropine, hyoscine hydrobromide did not modify the action of adrenaline on the vessels of the dog leg or on the nictitating membrane of the cat, or depress the level of the blood pressure in etherised cats in the doses administered.

The present paper reports upon an action of adrenaline which is inhibited by relatively small doses of atropine sulphate. A comparison was made between the effects of atropine sulphate (*r*), *l*-hyoscine, *l*-hyoscyamine, *l*-homatropine, trasentin 6H and 2786 R.P. (neoantergan) upon adrenaline activity in the preparation described below. The effect of neoantergan on the action of adrenaline on the isolated uterus of pregnant rabbit, and on the isolated gut segment of rabbit and guinea-pig was investigated. The effect of neo-antergan, atropine and trasentin 6H on adrenaline action on the blood pressure of spinal cats was also tested. In some cases comparison was made between the effects of the spasmolytic agents on the actions of *l*-adrenaline (B.P.) and *l*-nor-adrenaline.³

Graham⁴ showed that the isolated duodenum of the duck or drake contracts on addition of adrenaline to the fluid in which the strip of gut is suspended. The tissue is stiff and shows little spontaneous movement. The ileum of the drake and all parts of the intestine of fowls and pigeons show more activity and relax on addition of adrenaline. As Barsoum and Gaddum⁵ have shown, the rectal caecum of the fowl is sensitive to adrenaline in a concentration of 10^{-6} . The bath in which the duck gut was suspended was kept at 38°C. and was of 100 ml. volume. Drugs were added in solution in 0.5 ml. of saline solution.

THE ACTION OF ADRENALINE ON THE ISOLATED DUODENUM OF THE DRAKE AND INTERFERENCE WITH IT BY SPASMOLYTIC AGENTS

Adrenaline in a concentration of 2×10^{-8} or more causes a transient but powerful contraction of the isolated duodenum of ducks. This contraction is usually but not always followed by a period of relaxation which may or may not be accompanied by inhibition of spontaneous movement, so that the response to adrenaline may be purely motor⁴ or biphasic (see Figure 2). In the three preparations tested the response to *l-nor*-adrenaline was purely motor. In the same concentration as *l*-adrenaline it produced a longer though less vigorous contraction of the gut. Acetylcholine also causes a contraction in this preparation, but is less potent in this respect than adrenaline. The effect of acetylcholine 10^{-7} is roughly equal to that of adrenaline 2×10^{-8} . Atropine sulphate in a concentration of 10^{-6} abolishes or prevents the effect of the acetylcholine while leaving the action of adrenaline but little modified. If, however, the concentration is raised 100 fold to 10^{-4} the motor effect of adrenaline 2×10^{-8} on the gut is prevented. This effect is shown in Figure 1.

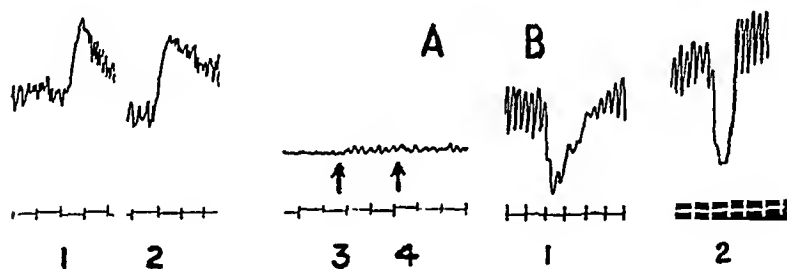


FIG. 1.—A. Isolated duodenum of duck. At 1. and 3. adrenaline 2×10^{-8} , at 2. and 4. acetylcholine 10^{-7} . Between 2. and 3. atropine sulphate 10^{-6} . B. Isolated rectal caecum of fowl. At 1. and 2. adrenaline 10^{-9} . Between 1. and 2. atropine sulphate 2×10^{-6} . Time in 10 secs.

The motor effect of adrenaline 10^{-7} is not inhibited by *l*-hyoscyne or *l*-hyoscyamine 10^{-5} , but is reduced to about one half by *l*-homatropine 10^{-5} and is abolished by atropine and trasentin 6H in the same concentration. The latter two compounds have a profoundly inhibitor effect on the tone and spontaneous movement of the gut in these high concentrations, the former have not. In the rectal caecum of the fowl the spontaneous movements are not inhibited by atropine (2×10^{-6}) nor is the inhibitor action of adrenaline (10^{-6}) modified by atropine in this concentration (see Figure 1).

The antihistamine compounds have been shown to have varying potencies as spasmolytic agents against contraction of smooth muscle caused by acetylcholine, histamine and barium (Graham⁶), and in the course of that work some inhibition of the effect of adrenaline on the

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blood pressure of the chloralosed cat was noted after addition of these compounds. Accordingly the effect of neoantergan (Dews and Graham⁷) was tested upon the action of adrenaline and *l*-nor-adrenaline on duck duodenum. As already stated the effect of adrenaline was to produce

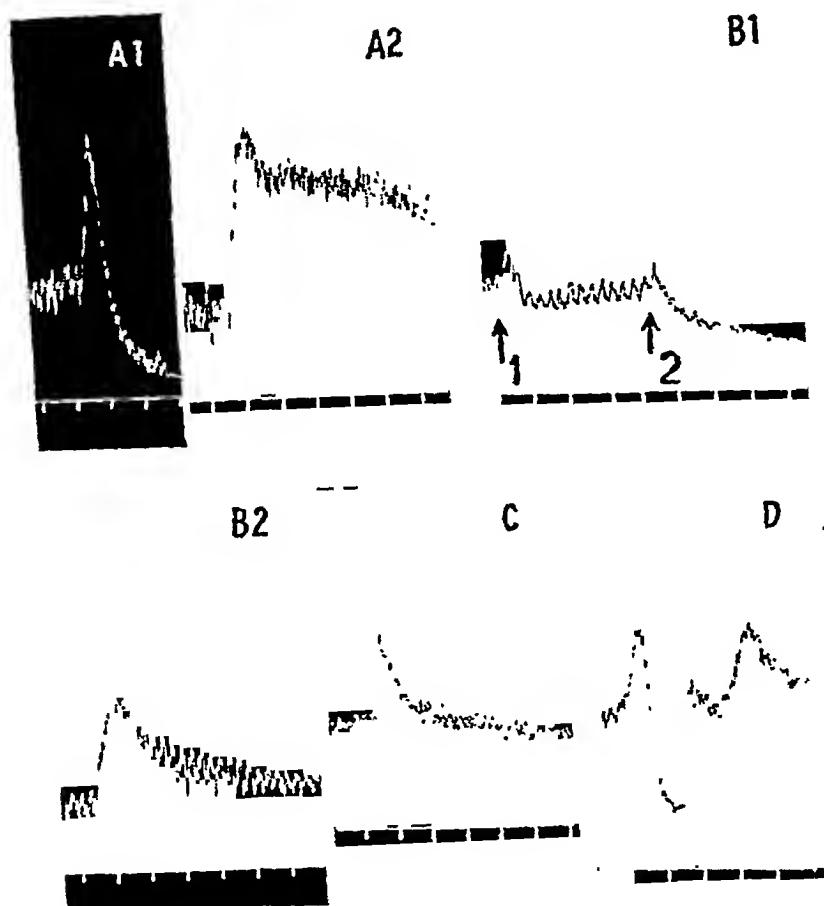


FIG. 2.—Isolated duodenum of duck. A1, biphasic response to *l*-adrenaline 10^{-7} . A2, motor response to *l*-nor-adrenaline 10^{-7} . B₁, neoantergan 10^{-6} injected at arrow 1, inhibits the motor part of the biphasic response to *l*-adrenaline 10^{-7} injected at arrow 2. B₂, as in A2 but in the presence of neoantergan 10^{-7} . C, effect of *l*-adrenaline 10^{-7} 5 minutes after neoantergan 10^{-6} had been washed out twice. Motor part of biphasic response only restored. D, as in A1 and A2 after 10 minutes washing out of neoantergan 10^{-6} . Time in 10 secs.

a biphasic response, that of *l*-nor-adrenaline to produce a motor response. Neoantergan in a concentration of 10^{-6} abolished the motor part of the response to adrenaline and reduced the inhibitor part of the response; the response to *l*-nor-adrenaline was partially inhibited (see Figure 2B). A concentration of 10^{-5} of neoantergan abolished the re-

sponse to either compound. The characteristic effect of both compounds could be restored after repeated washings, but the motor part of the response to adrenaline returned before the inhibitor portion of the response (see Figure 2C). These reactions are illustrated in Figure 2.

THE EFFECT OF NEOANTERGAN (2768 R.P.) ON THE RESPONSE TO ADRENALINE OF ISOLATED GUT OF RABBIT AND GUINEA-PIG, AND ISOLATED PREGNANT UTERUS OF RABBIT

In view of the inhibition by neoantergan of the action of adrenaline on the duodenum of ducks a similar trial of its effect on the action of adrenaline on isolated rabbit duodenum and guinea-pig duodenum was made. As is well known, the movement of such specimens is regular and is inhibited by adrenaline (10^{-8}). Preparations vary in the degree and duration of inhibition caused by this concentration of adrenaline. Neoantergan in a concentration of 10^{-5} usually caused the gut to relax and inhibited spontaneous movements; during this inhibition adrenaline caused no relaxation, but as the inhibition was already maximal no further effect could be expected. After repeated washings of the preparation the spontaneous contractions were small and frequent and the tone was high (Fig. 3F and 3H). Under these conditions in some preparations the action of adrenaline (10^{-8}) was abolished or reversed (3F) for a variable time, but ultimately returned. In other preparations (3H) the action of adrenaline was not abolished. These variations were related to the amount of neoantergan administered to the bath, the duration of its action, and to the period allowed for recovery and the number of wash-outs given. Inhibition of adrenaline action was better seen with specimens of duodenum than, of ileum.

Neoantergan in a concentration of 10^{-5} occasionally caused immediate spasm of the muscle preparation which lasted for some 2 minutes and was followed by increased spontaneous activity (3B). Under these circumstances the action of adrenaline could be tested in the presence of neoantergan (3B), when it was found to be less effective. After repeated washing the action of adrenaline was abolished (3C) and only partially restored after 10 washes at minute intervals (3D). Atropine, homatropine and trasentin had no effect on adrenaline inhibition of rabbit gut in low concentrations (10^{-8}); higher concentrations diminished gut movement so that further action by adrenaline could not be tested.

The isolated strip of pregnant uterus of rabbit responds to the addition of adrenaline (10^{-8}) with a transient spasm (Fig. 4A). Neoantergan in a concentration of 10^{-5} produces a prolonged increase in tone with frequent strong contractions superimposed upon it (4B). If adrenaline is added at the beginning or during the course of this period of increased tone caused by neoantergan the muscle is inhibited (4C and 4D). The action of adrenaline on this preparation is thus reversed by neoantergan in the concentration stated. Backman and Lundberg⁸ have shown that atropine has a similar effect on rabbit uterus.

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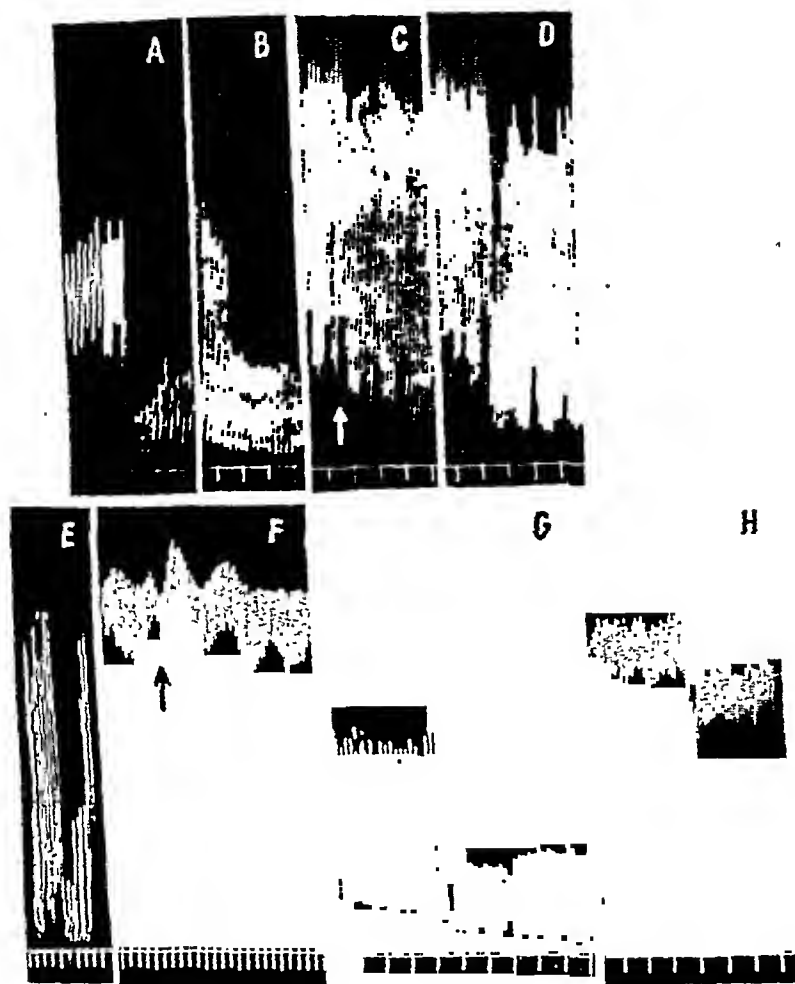


FIG. 3.—Isolated strips of rabbit duodenum. A. effect of adrenaline 10^{-8} . B. effect of A. in presence of neoantergan 10^{-2} . C. effect of A. after 5 changes of bath fluid at 1 minute interval. D. as in C. after 10 minutes interval. E. effect of adrenaline 10^{-8} on a fresh strip of duodenum. F. reversal of adrenaline effect 5 minutes after washing out neoantergan 10^{-2} . G. effect of adrenaline 10^{-8} on a fresh strip of duodenum. H. failure to modify action of adrenaline 5 minutes after neoantergan 10^{-2} . Contrast with E. and F. Time in 10 secs.

THE EFFECT OF ATROPINE, HYOSCINE, TRASENTIN AND NEOANTERGAN ON THE ACTION OF ADRENALINE ON THE BLOOD PRESSURE OF SPINAL CATS

Bussel² illustrates the fall in blood pressure in etherised cats which results from injection of atropine, and the inhibition of the response of the blood pressure to stimulation of the thoracic sympathetic chain and of the contraction of the nictitating membrane after administration of adrenaline to spinal cats, following upon injection of atropine.

: actions.

In a series of spinal cats adrenaline and *l*-nor-adrenaline were injected intravenously and the similar effects recorded. A comparison of the pressor effects of the two compounds on spinal cats indicated that *l*-nor-adrenaline has 165 per cent. of the pressor activity of *l*-adrenaline (B.P.) which agrees closely with the assay carried out by Tainter *et al*³ on dogs. It was found that injection of neoantergan 0.5 mg./kg. caused a transient small rise in blood pressure and slightly potentiated the

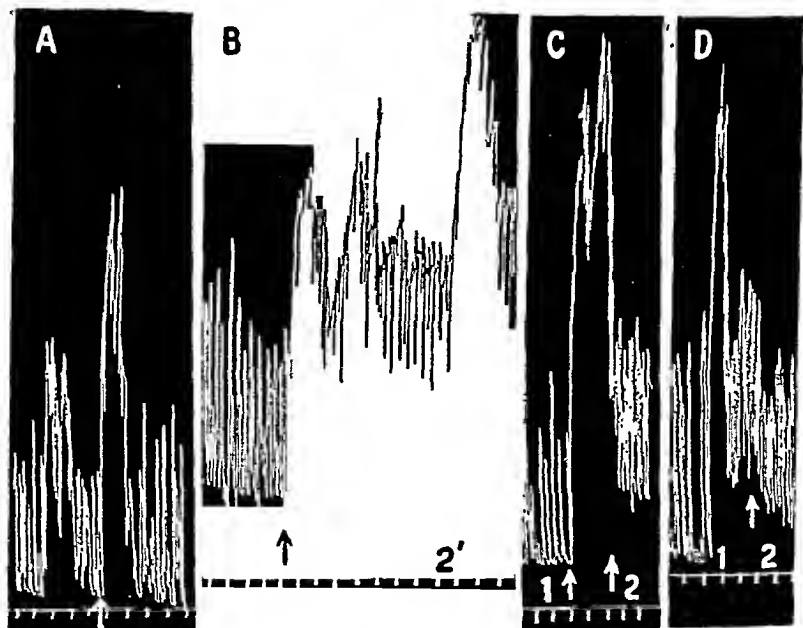


FIG. 4.—Isolated uterus of pregnant rabbit. A. motor effect of adrenaline 10^{-8} . B. prolonged motor effect of neoantergan 10^{-6} . C. adrenaline 10^{-8} added at arrow 2 inhibits the spasm caused by neoantergan 10^{-5} added at arrow 1. D. the effect of adrenaline 10^{-8} added at arrow 2 is reversed by previous addition of neoantergan 10^{-5} at point 1. Time in 2 minutes intervals.

action of these compounds (5B), while a dose of 4.0 mg./kg. caused a transient fall in blood pressure and inhibited the action of these compounds. This effect was brief. 8.0 mg./kg. caused a profound fall in blood pressure and further blocked the action of adrenaline and arterenol. Higher doses were too toxic to allow of further tests; benadryl and antistine⁶ cause a sharp fall in the blood pressure of chloralosed cats into which they are injected intravenously in doses of 1.0 mg./kg. Similar action has been cited by Bussel² as evidence of anti-adrenaline activity by atropine sulphate.

Trasentin 6H in a dose of 1.0 mg./kg. slightly potentiates the pressor effect of adrenaline or *l*-arterenol, while 4.0 mg./kg. inhibits the response. Larger doses of trasentin 6H (8.0 mg./kg.) completely suppress the response to adrenaline and *l*-arterenol, but are toxic, and under such

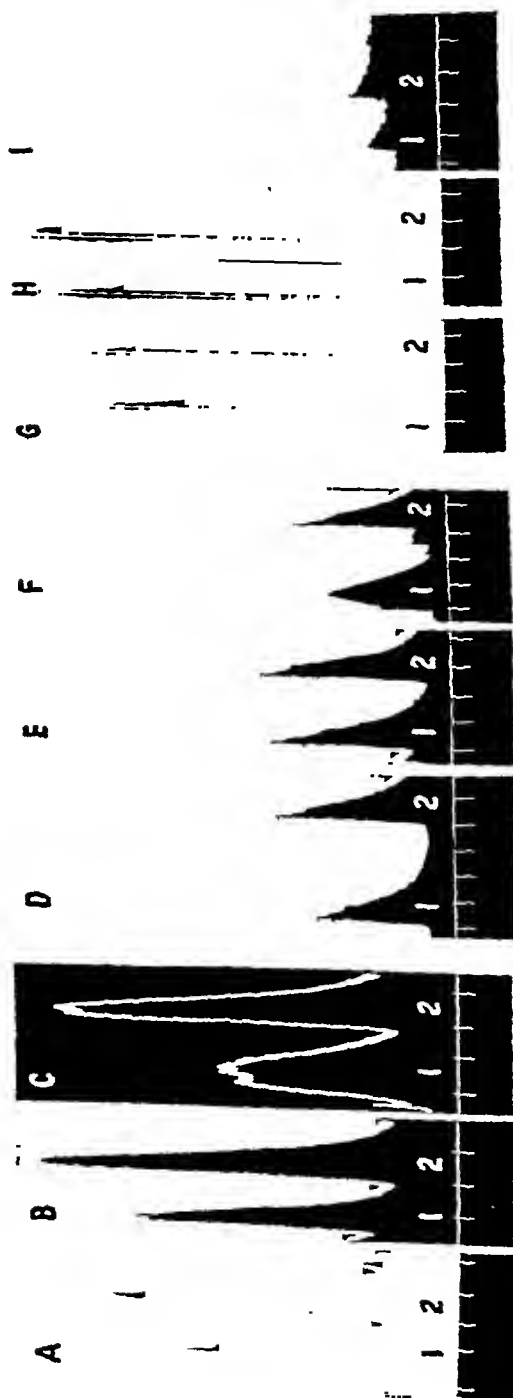


FIG. 5.—Carotid blood pressure taken from spinal cats of 2.5 to 3.5 kg. wt. Time in 30 sec. A1. pressor effect of *l*-nor-adrenaline 1.0 μ g./kg. A2. pressor effect of *l*-adrenaline 2.0 μ g./kg. B1. and 2. as above after neoatrogen 0.5 mg./kg. showing potentiation of effect of A1. and A2. C1. effect as in A1. inhibited after neoatrogen 4.0 mg./kg. C2. effect as in A2. still potentiated. Reversal of the order of administration of C1. and C2. reverses this picture which is due to the transient action of 4.0 mg./kg. of neoatrogen in blocking adrenaline activity. D1. and D2. as in A1. and A2. but different cat. E1. and E2. as in D1. and D2. but after trasentin 1.0 mg./kg., effect potentiated. F1. and F2. as in D1. and D2. but after trasentin 4.0 mg./kg.; effect inhibited. G1. and G2. *l*-nor-adrenaline 1.0 μ g./kg. and *l*-adrenaline 1.6 μ g./kg. respectively. H1. and H2. as in G1. and G2. but after atropine sulphate 1.0 mg./kg., effect inhibited. I1. and I2. as in G1. and G2. but after atropine sulphate 20 mg./kg., effect inhibited.

conditions the failing heart and low blood pressure are incapable of responding to any stimulus.

l-Hyoscine (1.0 mg./kg.) slightly potentiates the effect of adrenaline and *l*-nor-adrenaline, but has no further effect in doses up to 8.0 mg./kg. Atropine sulphate 1.0 mg./kg. slightly potentiates the effect of *l*-adrenaline and *l*-nor-adrenaline, 4.0 mg./kg. has a stronger action, and 20.0 mg./kg. almost abolishes the effect of both compounds without undue toxic actions on the cardio-vascular system of the cats (51).

In drakes anaesthetised with pentobarbitone solution injected into the breast muscles followed by inhalation of ether, the administration of atropine sulphate 1.0 mg./kg. intravenously causes a sharp transient fall in blood pressure of about 20 mm. Hg. This is similar to the effect seen in etherised cats and rabbits. Doses of 10.0 mg./kg. of atropine do not inhibit the pressor effect of adrenaline 10 µg./kg. intravenously in this preparation.

DISCUSSION

Much recent work has increased our knowledge of the close and complex relationships between the functions of the sympathetic and parasympathetic nervous systems and the actions of acetylcholine and adrenaline and other compounds which modify their activity. The effect of such a substance on any organ is closely related to the dose administered. McDowall⁹ and Elio¹⁰ have shown that small doses of acetylcholine may stimulate the heart; larger doses inhibit it. Acetylcholine may potentiate the effect of adrenaline on the heart and blood vessels, and the opposite may occur in the central nervous system (Burn¹²). Small doses of adrenaline lower the blood pressure, larger doses raise it. Many sympathomimetic compounds such as ephedrine, amphetamine, tyramine, cocaine and other local anaesthetics have a different effect on the activity of adrenaline according to the concentration in which they are administered (Jang¹³, Graham and Gurd¹⁴).

Sherif¹⁵ has shown that the hypogastric nerve to the uterus of the bitch is cholinergic in nature but is not paralysed by atropine, while Secker¹⁶ has shown that salivary secretion in the dog following upon injection of adrenaline and sympathetic stimulation is inhibited by atropine. The inhibitory action of atropine on the effect of adrenaline on duck duodenum is moderately potent (atropine 10^{-6} is effective), while the inhibitory action of trasentin 6H and homatropine is less effective (10^{-5}) and *l*-hyoscine and *l*-hyoscyamine appear to be relatively ineffective. There is no apparent relation between the activity of these compounds in inhibiting the action of adrenaline on this preparation and their relative potencies in inhibiting the action of acetylcholine (Graham and Gunn¹⁷). No action could be shown of these spasmolytic compounds in preventing the inhibitor effects of adrenaline in isolated rabbit gut since in effective doses they are themselves powerful inhibitors of spontaneous movement in this preparation. While atropine and trasentin 6H in doses of 1.0 mg./kg. produce a transient fall in blood pressure

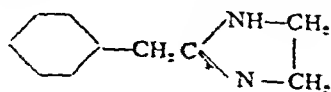
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in cats which have a good vascular tone and a high blood pressure (ether or chloralose anæsthesia) it takes much greater quantities of these drugs to inhibit the pressor effect of adrenaline in the spinal cat. It may be therefore that the transient fall in blood pressure seen in etherised cats after atropine, trasentin, etc., 1.0 mg./kg. is a non-specific spasmolytic effect on the arteriolar muscle in high tone rather than a specific anti-adrenaline effect as Bussel² suggests, but the clear diminution in the pressor response to stimulation of the thoracic sympathetic chain in spinal cats shown by that author and the great inhibition of the pressor response to injected adrenaline and *nor*-adrenaline illustrated in Figure 5 of this report show that atropine exerts a blocking effect on the motor actions of adrenaline, as does trasentin 6H. Bussel² attributes this blocking effect of atropine to its structural resemblance to cocaine which when present in low concentration competes with adrenaline for receptors on the enzymes which inactivate adrenaline and thus potentiates the effect of the latter, and in high concentrations competes with adrenaline for cell receptors and thus inhibits its action (MacGregor¹⁸). In support of Bussel's explanation of atropine in high concentration inhibiting adrenaline activity is the finding that doses of 1.0 mg./kg. (1/20 of the adrenaline-inhibiting dose) increased the pressor response to adrenaline, as did *l*-hyoscine, trasentin 6H and neoantergan.

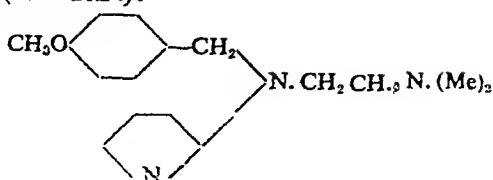
Neoantergan^{6,7} inhibits spasm of smooth muscle caused by histamine, acetylcholine and barium. It also blocks the relaxation of some but not all smooth muscle caused by histamine. It appears from the experiments described that it not only inhibits the pressor effect of adrenaline on the vascular bed but reverses the motor effect on the uterus, and inhibits the relaxor effect of adrenaline on some preparations of gut. Its action in this respect differs from that of dibenamine¹⁹ which can reverse the motor response of rabbit uterus and the pressor response of the cat to administration of adrenaline, but does not affect the inhibitory action of adrenaline on gut. The properties of adrenolysis and sympatholysis are shown in varying degree by ergot²⁰, yohimbine²¹, 2-diethyl-aminoethyl-1:4-benzdioxan (833F) and 2-piperidinomethyl-1:4-benzdioxan (933F), ephedrine, cocaine, atropine, trasentin 6H, neoantergan, priscol²², etc. Of these compounds only priscol has been stated to show the property described for neoantergan of blocking the inhibitory action of adrenaline on some segments of rabbit gut. Ahlquist *et al*²² do not consider this a specific effect of priscol because of the irregularity of appearance of the phenomenon.

Adrenaline may be written as $R_1\text{.CHOH.CH.NH.R}_2$. A similar ethylamine chain may be visualised with varying degrees of ease in all the adrenolytic compounds mentioned.

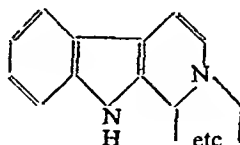
In dibenamine it appears thus: $\text{—Cl.CH}_2\text{.CH}_2\text{.N(CH}_2\text{.Ph)}_2$.
in priscol: —



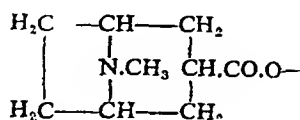
in neoantergan (2786 R.P.):—



-in yohimbine²³:—



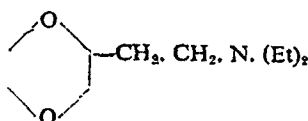
in atropine:—



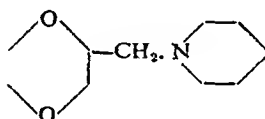
in trasentin 6H:—



in 833 F:—



and 933 F:—



The possession of a common element of structure would account for common properties on the basis of such a theory as that propounded by MacGregor¹⁸ for the effect of cocaine on adrenaline action.

That the effect of spasmolytic compounds in blocking adrenaline action is essentially similar to their effect in blocking *l*-nor-adrenaline is of interest in view of the suggestion of Euler²⁴, Bacq and Fischer²⁵ and Gaddum and Goodwin²⁶ that *nor*-adrenaline is sympathin. West²⁷ compared *l*-adrenaline and *dl*-*nor*-adrenaline, and found that both had a powerful inhibitory effect on isolated segments of rabbit gut. The same effects were found above with *l*-*nor*-adrenaline but in duck gut, in which *l*-adrenaline produces a biphasic response, *l*-*nor*-adrenaline is purely motor.

SUMMARY

1. Isolated segments of duodenum from the duck react to the addition of adrenaline by a biphasic response, contracting and relaxing. Atropine, trasentin 6H, homatropine and neoantergan (2786 R.P.)

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abolish this contractile response. Hyoscine and hyoscyamine have no effect in concentrations of 10^{-5} .

2. Rabbit gut is inhibited by adrenaline and *l-nor*-adrenaline. This action is abolished after neoantergan has been in contact with some segments of gut.

3. Rabbit uterus contracts with adrenaline or neoantergan. In the presence of the latter the former inhibits movement of the uterus.

3. The pressor response to adrenaline and *l-nor*-adrenaline in spinal cats is inhibited by atropine, trasentin and neoantergan in high doses (atropine sulphate 20.0 mg./kg.).

4. Certain differences between *l*-adrenaline and *l-nor*-adrenaline are discussed.

5. A tentative hypothesis is offered to account for the adrenolytic activity found in a wide variety of compounds. This is an extension of MacGregor's well-known explanation for the reactions of cocaine and adrenaline, based on certain common structural features in the adrenolytic agents discussed.

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THE PREPARATION AND RESOLUTION OF ACRIDYL(5)-N-ALANINE ETHYL ESTER

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IT HAS BEEN REPORTED that D amino-acids are present in the molecules of various antibiotic polypeptides such, as gramicidin^{1,2}, tyrocidin³, gramicidin S⁴, aerospornin⁵ and bacitracin⁶. The penicillins on degradation give D-penicillamine (β : β -dimethylcysteine) and du Vigneaud and his collaborators⁷ have shown that an antibiotic penicillin may be synthesised from D-penicillamine, whereas the isomer from L-penicillamine is biologically inactive.

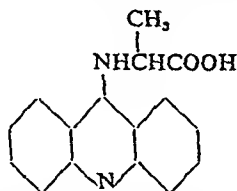
Several workers have prepared substances containing D amino-acids for study as antibacterial agents. These substances either represented possible fragments of the antibiotic molecules, or were of closely related structure. Harris and Work⁸ prepared two open chain pentapeptides containing the five amino-acids of gramicidin S in the sequence suggested for the amino-acids in the antibiotic molecule⁹. In one of the pentapeptides the phenylalanine possessed the L configuration; in the other it had the D configuration as in the antibiotic. No significant difference in antibacterial action was found between the two pentapeptides. Fruton¹⁰ synthesised the diketopiperazine from D-leucyl-L-tryptophane because gramicidin had an unusually high content of these two amino-acids; the compound possessed no antibacterial activity. D-leucine and D-valine have been isolated from gramicidin hydrolysates and Fling, Minard and Fox¹¹ synthesised the prolyl derivatives of both D- and L-valine and D- and L-leucine. Proline was chosen as the second component of the dipeptides because gramicidin contained no free amino groups. No antipodal specificity was observed in the growth inhibitory actions of the dipeptides and a similar result was observed for the corresponding phthalyl derivatives. The four diastereoisomeric leucyl-leucines were prepared by Fox, Kobayashi, Melvin and Minard¹², who considered D-leucyl-D-leucine of especial interest because of its relationship to D-valyl-D-valine, which had been isolated from partial hydrolysates of gramicidin. No appreciable activity was found in any of the dipeptides. Linnell and Smith¹³ synthesised DL-N β -hydroxyethylalanine which combined the essential structural features of both alanine, the simplest amino-acid exhibiting optical isomerism, and also ethanolamine which had been isolated from gramicidin hydrolysates. The racemic compound possessed no growth inhibitory properties.

These results indicate that growth inhibitory properties are not concomitant with the presence of D amino-acids in a molecule. The available evidence supports the opinion of Work¹⁴ that antibiotics containing D amino-acids are active, not because they have this character in common, but rather in virtue of their individual structures, of which the D amino-

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acid must be regarded as an integral structural part. The toxicity of these antibiotics may be due to the possession of cyclic structures, such as have been suggested for gramicidin S and tyrocidin¹⁴, or to some hitherto undiscovered features of the molecules. The mere presence of a D amino-acid in a molecule does not seem to be sufficient for the production of antibacterial properties in that molecule and some other structural feature or features appear to be necessary. An illustration of this consideration is the contrast between the thiazolidine- β -lactam system of the penicillins and the structure of D-penicillamine which has no antibacterial properties.

It therefore appeared of interest to prepare the optical isomers of a substance which combined the structures of an amino-acid and an established antibacterial nucleus. Comparison of the antibacterial activities of the two isomers would afford information relating to the importance of configuration of the amino-acid residue in such compounds. 5-Aminoacridine was chosen as the antibacterial nucleus and the compound DL-acridyl(5)-N.alanine was prepared in 96 per cent. yield, as a yellow powder, m.pt., 214°C., by the condensation of 5-chloracridine with DL-alanine in phenol solution.



Bacteriological testing of this compound was not possible because of its insolubility and the instability of its salts in aqueous media. A similar result had been reported for acridyl(5)-N.glycine by Dupré and Robinson¹⁵. Esterification with ethyl alcohol in the presence of dry hydrochloric acid gas gave an 80 per cent. yield of the racemic ethyl ester as small yellow prisms, m.pt. 75°C. The ester gave well defined crystalline compounds with picric and picrolonic acids and its diacetate and monohydrochloride were quite stable in aqueous solutions. A 0.1 per cent. aqueous solution of the diacetate possessed growth inhibitory activity against *Staphylococcus aureus* and *Streptococcus pyogenes*.

The racemic ester was resolved by the use of (+) tartaric acid. Hot absolute alcoholic solutions, containing equivalent quantities of the ester and (+) tartaric acid, on mixing gave an 87 per cent. yield of the racemic ester (+) tartrate. This salt was a deep yellow powder, m.pt., 165° C. $[\alpha]_D^{20} + 18^\circ$ (water, $c = 4.0$). Fractional crystallisation from alcohol (80 per cent.) gave the (+) ester (+) tartrate as long yellow needles, m.pt., 118° C. $[\alpha]_D^{20} + 54.5^\circ$ (water, $c = 4.0$). Evaporation of the mother liquors and repeated crystallisation of the residue from

compound in 10 ml. of alcohol. Yellow powder. Yield: 0.25 g. m.pt., 194°C. Found: C, 55.0; H, 4.07; N, 13.0 per cent. $C_{24}H_{21}N_5O_9$ requires C, 55.1; H, 4.0; N, 13.37 per cent.

(d) *Styphnate*.—Light yellow powder. Yield: 0.2 g. m.pt., 178°C. Found C, 53.1; H, 3.8; N, 13.2 per cent. $C_{24}H_{21}N_5O_{10}$ requires C, 53.4; H, 3.9; N, 13.0 per cent.

(e) *Picrolonate*.—Yellow powder. Yield: 0.25 g. m.pt., 227°C. (decomposed). Found: C, 59.4; H, 4.5; N, 14.2 per cent. $C_{27}H_{26}N_6O_8$ requires C, 57.5; H, 4.6; N, 14.9 per cent.

4. Resolution of DL-Acridyl(5)-N.alanine ethyl ester.

(a) DL-Acridyl(5)-N.alanine ethyl ester (+) tartrate.—8 g. of the racemic ester and 4 g. of (+) tartaric acid were dissolved in 100 ml. of hot absolute alcohol. On cooling small yellow crystals appeared. Yield: 10.5 g. (87 per cent.), m.pt., 165°C. (decomposes with effervescence), $[\alpha]_D^{20^\circ} + 18^\circ$ (4 per cent. solution in distilled water). Found: C, 57.7; H, 5.65; N, 6.2 per cent.; $C_{22}H_{24}N_2O_8$ requires C, 59.4; H, 5.4; N, 6.3 per cent. The optical rotation and melting-point remained unchanged after several recrystallisations from alcohol, but as the substance was easily soluble in water, fractional recrystallisation was attempted from a series of aqueous alcohols. It was found that alcohol (80 per cent.) caused the preferential separation of the (+) ester (+) tartrate.

(b) (+) Acridyl(5)-N.alanine ethyl ester (+) tartrate.—20 g. of the racemic ester (+) tartrate were dissolved in 400 ml. of hot aqueous alcohol (80 per cent.). Long yellow needle crystals slowly formed and these were filtered after 48 hours' standing. Yield: 7.1 g. m.pt., 118°C. $[\alpha]_D^{20^\circ} + 54.5^\circ$ (4 per cent. solution in distilled water). The melting-point and optical rotation remained constant after several recrystallisations from the same solvent and it was considered that this material was the (+) ester (+) tartrate.

(c) (+) Acridyl(5)-N.alanine ethyl ester.—2 g. of the (+) tartrate salt were dissolved in 50 ml. of distilled water, 30 ml. of benzene was added and then 1 per cent. aqueous ammonia drop by drop. After each addition of the ammonia the yellow precipitate formed was shaken into the benzene. When no further precipitation occurred the benzene layer was separated and dried for 24 hours over anhydrous sodium sulphate. Removal of the solvent under reduced pressure left a yellow viscous oily residue. Yield: 1.05 $[\alpha]_D^{20^\circ} + 128^\circ$ (2 per cent. solution in alcohol (96 per cent.)). Found: C, 74.4; H, 6.5; N, 9.02 per cent. $C_{18}H_{18}N_2O_2$ requires C, 73.4; H, 6.12; N, 9.5 per cent. The (+) ester gave a crystalline picrate, m.pt., 175°C. (m.pt. of racemic ester picrate, 194°C.) Found: C, 54.5; H, 3.65; N, 13.3; $C_{24}H_{21}N_5O_9$ requires C, 55.1; H, 4.0; N, 13.37 per cent.

The use of chloroform instead of benzene in the preparation caused complete racemisation of the ester.

(d) (–) Acridyl(5)-N.alanine ethyl ester (+) tartrate.—The mother liquid from the crystallisation of the (+) ester (+) tartrate was allowed

to remain at room temperature for a further 48 hours and 1.5 g. of a yellow powder, m.pt., 111° to 113°C., $[\alpha]_D^{20} + 30.5^\circ$, was obtained by filtration. The solvent was removed from the filtrate and the yellow viscous residue repeatedly crystallised from absolute alcohol until a constant value for the optical rotation was obtained. The material formed a yellow powder which did not possess a sharp melting-point, the substance softened gradually from 80°C. onwards. $[\alpha]_D^{20} - 19.5^\circ$ (4 per cent. solution in distilled water.)

(e) (-) *Acridyl(5)-N-alanine ethyl ester*.—This was isolated from the (-) ester (+) tartrate by a similar procedure to that used for the (+) ester. It was a yellow viscous oil, $[\alpha]_D^{20} - 122^\circ$ (2 per cent. solution in alcohol (96 per cent.)).

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PALTHÉ SENNA AS AN ADULTERANT OF INDIAN SENNA LEAVES

By J. L. FORSDIKE

Received September 27, 1948

DURING recent months, a considerable quantity of Indian senna leaves adulterated with a foreign leaf has been offered for sale in this country. This adulteration has ranged from 5 to 10 per cent. in better quality leaves to upwards of 90 per cent. in some cheaper samples. The adulterant has been identified, by comparison with authentic specimens from the Herbarium of the Pharmaceutical Society of Great Britain, as the leaf of *Cassia auriculata* Linn., commonly known as Palthé Senna. The substitution of this leaf for senna has been previously reported¹, a colour reaction for its detection has been suggested by Vamossy² and some description of the leaves is given in Thoms' Handbuch³ and by Wasicky⁴. However, since this adulteration appears to be occurring on a somewhat extensive scale, it was thought that some more detailed investigation, particularly of the anatomy of Palthé senna might be of value.

MATERIAL

The following description is based on leaflets taken from two sheets in the Herbarium of the Pharmaceutical Society of Great Britain marked:—

- (1) *Cassia auriculata*, Deccan. Dr. T. Cooke
- (2) *Cassia auriculata*, January, 1888. Rajputana. Coll. J. G. Prebble, and on leaflets picked out from nine commercial samples, offered for sale as Tinnevely senna.

MACROSCOPICAL CHARACTERS

The leaflets are oblong or obovate and generally smaller than those of genuine senna, being 8 to 30 mm. long and 4 to 15 mm. wide, yellowish to greyish-green, sometimes with a purple tinge; they are thin and brittle; the margin is entire; the apex blunt and mucronate and the base unequal, with a very short stalk (Fig. 1, A.). Both surfaces of the leaf bear numerous hairs, visible under a hand lens; the veins are more prominent on the under surface.

MICROSCOPICAL CHARACTERS

Upper Epidermis. Polygonal prisms, with nearly straight anticlinal walls and a thin cuticle; many cells containing mucilage attached to the inner periclinal wall; this mucilage staining with ruthenium red. (Fig. 1, B, ep.; C.) Stomata, 100 to 200 per sq. mm., sunk below the general epidermal level, rubiaceous, commonly having one subsidiary cell much larger than the other. (Fig. 1, B, C, st.) Trichomes 130 to 240 to 650 to 830 microns long and 14 to 22 microns wide, conical, unicellular, thick-walled, with a pointed apex and only very slightly warty cuticle. (Fig. 1, B, D, t.)

Lower Epidermis. Similar to the upper, except that the cells are generally rather smaller, the anticlinal walls are sometimes slightly wavy and the stomata are more numerous, 130 to 260 per sq. mm. (Fig. 1, B, ep₂:D.).

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Mesophyll. Dorsiventral, the palisade on the upper surface consists of two layers of cells, the cells of the upper layer being very elongated, up to ten times as long as they are wide. (Fig. 1, B, p_1 , p_2 .) The spongy mesophyll consists of 3 to 4 layers of cells, the lowermost layer being often slightly elongated at right angles to the epidermis. (Fig. 1, B, s.)

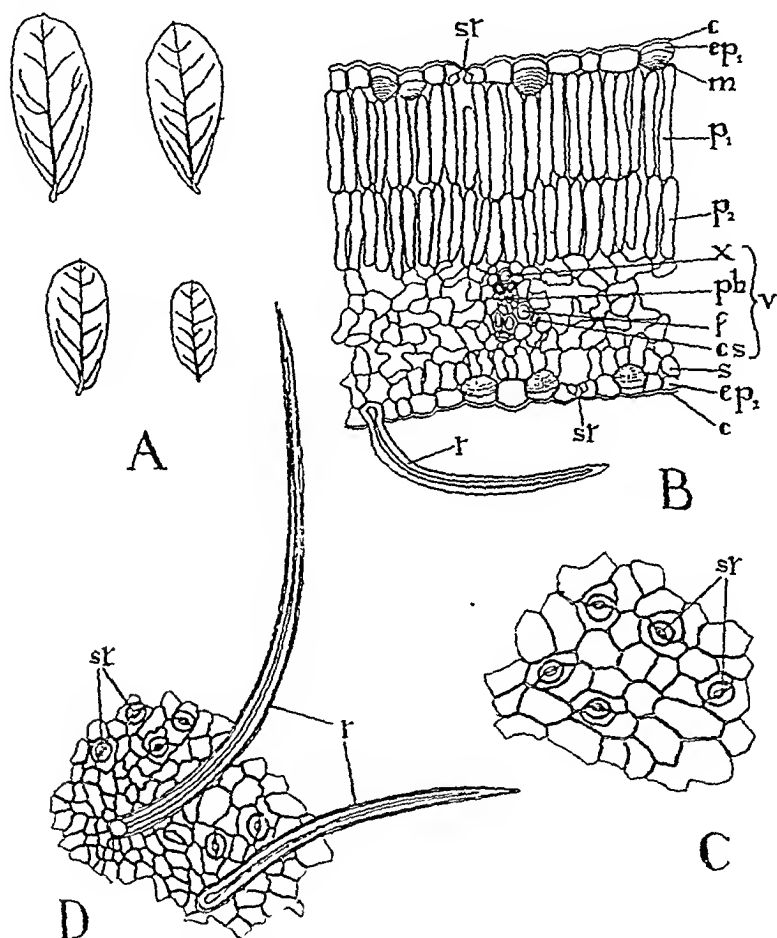


FIG. 1. *Cassia auriculata*.—A, whole leaflets, natural size; B, transverse section of lamina; C, upper epidermis, surface view; D, lower epidermis, surface view; c, cuticle; ep₁, upper epidermis; ep₂, lower epidermis; m, mucilage; p, upper layer of palisade; p₂, lower layer of palisade; x, xylem; ph, phloem; f, fibres; cs, crystal sheath; s, spongy mesophyll; st, stomata; t, trichomes; v, veinlet. All x 150, except A.

Cluster crystals of calcium oxalate, 6 to 8 to 15 to 20 microns in diameter occur, but are not common, being usually found along the main veins and most frequent in the neighbourhood of the midrib. Both layers of palisade are continuous over the midrib.

Stelar Tissues. The midrib consists of a radiate xylem, containing

annular, reticulate and pitted vessels, below which are groups of sieve tissue. There is an arc of pericyclic fibres below the phloem and a group of fibres above the xylem. The fibres are surrounded by a sheath of cells, each containing a single prism of calcium oxalate, 7 to 10 to 20 to 24 microns long by 4 to 5 to 8 to 10 microns wide. Both xylem vessels and fibres have lignified walls. The midrib projects slightly on the under side of the leaf, the projection being filled with collenchyma.

The large veins form a network and are accompanied by a complete crystal sheath, similar to that described for the midrib. In the smaller veinlets the complete sheath is lacking, but occasional prisms of calcium oxalate are found along these veinlets. (Fig. 1, B, v.)

QUANTITATIVE DATA

Stomatal Index. The stomatal indices of Palthé senna and of the official sennas are:—

Cassia auriculata 7.1 to 9.0 to 13.0 to 14.5

Cassia acutifolia 8.1 to 10.0 to 14.0 to 15.5 (Rowson⁵)

Cassia angustifolia 15.6 to 16.0 to 21.5 to 22.7 (Rowson⁵).

The stomatal index will thus serve to distinguish Palthé senna from Indian senna, but not from Alexandrian senna.

Vein-islet Number. The vein-islets numbers of the three sennas are:—

Cassia auriculata 18 to 26.

Cassia acutifolia 25 to 20 (Wallis⁶)

Cassia angustifolia 20 to 23 (Wallis⁶).

This feature is thus available to distinguish *C. auriculata* from Alexandrian senna, but will not give a distinction from Tinnevely senna.

Palisade Ratio. The palisade ratio of *Cassia auriculata* was determined under the same conditions as those used by George⁷ for genuine senna. The results are:—

Cassia auriculata 2.5 to 4.5 to 8.0

Cassia acutifolia 3.5 to 8.0 to 18.0 (George⁷)

Cassia angustifolia 2.5 to 5.6 to 12.0 (George⁷).

George reports that an average of 20 determinations on Alexandrian senna will give a result of over 7.5. On Palthé senna an average of 20 determinations may be expected to give a result of less than 6.0, so that palisade ratio determinations serve as a ready means of distinguishing these two species. The values obtained for *C. auriculata* and *C. angustifolia* are, however, too close for palisade ratios to be of much value in differentiating between these species.

COLOUR REACTIONS

Sulphuric Acid (80 per cent. v/v). As first reported by Vamossy², Palthé senna leaves give a crimson colour with 80 per cent. v/v sulphuric acid. This test may be carried out by sprinkling the broken leaves, or powder, on to the surface of the acid in a test-tube. When examining powdered senna for possible adulteration with Palthé senna, the test is best carried

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out by mounting the powder in 80 per cent. v/v sulphuric acid and examining microscopically. Treated in this way, particles of Palthé senna show a brilliant crimson colour and 2 per cent., or less, of this leaf may be detected in admixture with genuine senna.

Chloral Hydrate. Heating Palthé senna leaves, whole or in powder, with solution of chloral hydrate (5 in 2), in a boiling water-bath also results in a crimson colour, changing to brownish-red on longer heating.

Bornträger's Reaction. Palthé senna leaves give no rose-red colour with Bornträger's reaction, either direct or after hydrolysis by boiling with dilute sulphuric acid. It was concluded from this that the leaves contain no anthraquinone derivatives; a fact which is confirmed by Wasicky⁴. It should be noted, however, that Maurin⁸ reported finding 0.7 per cent. of oxymethylantraquinones in the leaves of *C. auriculata* and 1.9 per cent. in the stem bark.

SUMMARY

The principal characters which serve to differentiate Palthé senna from the official sennas, particularly in the broken or powdered condition, are:—

1. The trichomes, which are about three times as long as those of genuine senna and lack a conspicuously warty cuticle.

2. The presence of two layers of palisade cells on the upper side of the leaf and none on the lower.

3. The cluster crystals being usually along the main veins, whereas the clusters of genuine senna are not found in the neighbourhood of the veins.

4. The presence of occasional prisms of calcium oxalate on the smaller veinlets, where they are lacking in the official sennas.

5. The palisade ratio, stomatal index and vein-islet number.

6. The colours produced by 80 per cent. v/v sulphuric acid and chloral hydrate.

7. The absence of anthraquinone derivatives, resulting in the leaf giving a negative result with Bornträger's reaction.

I wish to thank Dr. T. E. Wallis, the Curator of the Pharmaceutical Society's Museum, for the supply of authentic material and also for calling my attention to some of the literature cited. I am indebted to the Directors of Boots Pure Drug Company, Ltd., in whose Analytical Laboratories the work was carried out, for permission to publish this paper.

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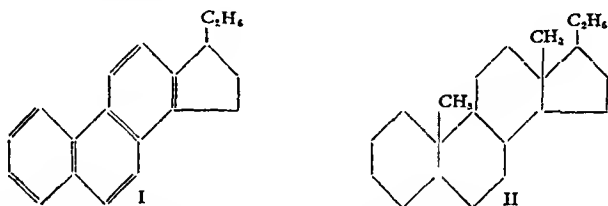
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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Conessine, Constitution of. R. D. Hayworth, J. McKenna and N. Singh. (*Nature*, 1948, 162, 22.) Careful fractionation of the selenium dehydrogenation products of the hydrocarbon mixture $C_{21}H_{36}$, from pyrolysis of conessine dihydriodide, yielded a crystalline hydrocarbon $C_{21}H_{36}$, m.pt. 78° to $79^\circ C.$, which showed many similarities with synthetic 3-ethylcyclopentenophenanthrene (I), m.pt. 84.5° to $85.5^\circ C.$, although the identity was not convincingly established.



Degradation of conessine by the Hofmann and Emde processes gave a hydrocarbon $C_{21}H_{36}$ which on reduction yielded approximately equal amounts of two isomeric hydrocarbons $C_{21}H_{36}$, m.pt. 56° to $58^\circ C.$ and 83° to $84^\circ C.$ The latter isomer is identical chemically and physically with *allopregnane* (II) prepared from progesterone. The carbon atoms of conessine are therefore accounted for by the *allopregnane* structure (II) and the three N-methyl groups, but the positions of the ethylenic linkage and the points of attachment of the basic centres are still uncertain. R. E. S.

Iodosalicylates of Alkaloids. M. Covelio and A. Capone. (*Ann. Chim. appl., Roma*, 1948, 38, 123.) 3-Iodosalicylic acid and 5-iodosalicylic acid were compared with picric acid and styphnic acid as reagents for the detection of alkaloids. In general the solubilities of the salts are greater than those of the corresponding picrates and styphnates, that is, the reaction is less delicate. Pilocarpine did not precipitate in 10 per cent. solution and codeine only in solutions stronger than 0.7 per cent; these salts may be useful for hypodermic use. The 3-iodosalicylates of brucine (m.pt. $224^\circ C.$ decomp.), quinine (m.pt. neutral 164° to $164.5^\circ C.$, basic $156^\circ C.$ decomp.) cinchonine (m.pt. 167.5° to $168^\circ C.$) morphine (m.pt. 194° to $194.5^\circ C.$ decomp.) and strychnine (decomposes at about $192^\circ C.$) were prepared and illustrations of the microscopic appearance of the crystals, which may be useful for identification of the alkaloids, are given.

H. D.

ANALYTICAL

Adrenaline, Determination of, with Iodine. J. E h r l e n. (*Farm. Revy*, 1948, 47, 321.) The photometric determination is usually carried out at pH 4 to 7. At a higher pH the colour rapidly fades owing to further oxidation; at a lower pH the colour development is very slow. By using oxidising agents such as potassium ferricyanide, adrenochrome is produced with an absorption maximum at $485 m\mu$. With iodine, however, a mixture of adrenochrome and iodoadrenochrome is obtained and the absorption maximum may be anywhere

between 485 and 525 $m\mu$. The more acid the solution, the greater the proportion of iodoadrenochrome. The mixed colour has sometimes been considered incorrectly as a variation of colour with the pH. In the method of Thorvik, carried out at pH 4.8, the adrenaline is converted almost quantitatively into iodoadrenochrome. Other oxidising agents simplify the course of the reaction; potassium ferricyanide is very suitable. At pH 6 the oxidation velocity is fairly high and nearly quantitative, corresponding to a calculated molar extinction coefficient of 4250 at 485 $m\mu$. The reaction of the solution is then adjusted to pH 3 to 4, when the adrenochrome is very stable. An advantage of this method is that, in presence of procaine, no precipitate is formed. When iodine is used, the extinction curves for adrenochrome and iodoadrenochrome cut one another at 525 $m\mu$ and at this point the result is independent of the proportions of the two compounds formed. G. M.

Adrenaline, Fluorimetric Determination of. J. E h r l e n. (*Farm Revy.*, 1948, 47, 242.) The method, which is applied particularly to procaine solutions, is a development of that recently published by the author, and the fluorescence is now measured photometrically, using a filter (maximum transmission at 405 $m\mu$; cut-off at 480 $m\mu$) in the incident light, and another filter (not transmitting below 500 $m\mu$) between the sample and the photo cell. The fluorescence results from atmospheric oxidation in alkaline solution, but is rapidly destroyed by further oxidation. It is suggested that the fluorescent compound is 1-methyl-3 : 5 : 6-trihydroxyindol, formed directly from adrenochrome. Under the conditions given by the author, the oxidation of adrenaline to adrenochrome is quantitative, and in the next stage the addition of a reducing agent prevents the further oxidation of the fluorescent compound during the assay. Details are as follows. A sample containing 3 to 30 μg . of adrenaline and 10 to 50 mg. of procaine is diluted with water to 3 ml. and mixed with 0.1 ml. of 0.1M hydrochloric acid and 0.5 ml. of 0.15M (2 per cent.) sodium acetate solution. To this is added 1.0 ml. of a 0.5 per cent. solution of potassium ferricyanide. After two minutes the mixture is made up to 25 ml. with a mixture of 5 ml. of 5M sodium hydroxide, 5 ml. of alcohol (95 per cent.), 10 ml. of water, and 0.5 ml. of a 5 per cent. solution of ascorbic acid. The solution is transferred to a sample holder, and the fluorescence is read off after 15 minutes. A blank on the reagents is also done. The standardisation should be carried out on solutions and under conditions similar to those of the assay. G. M.

Alkyl Nitrates, Determination of, in Pharmaceutical Preparations. P. L u n d g r e n and T. C a n b ä c k. (*Svensk farm. Tidskr.*, 1948, 52, 298, 313, 333.) Although the phenoldisulphonic acid method of determination of nitrates is in general satisfactory, it has certain limitations. In particular it is not suited for the determination of alkyl nitrates in oil solutions or in ointments. These limitations do not apply to *m*-xylenol-(4-hydroxy-1 : 3-dimethylbenzene), which is nitrated to *o*-nitroxylenol(5-nitro-4-hydroxy-1 : 3-dimethylbenzene). A photometric determination of the dissociation constant of the nitro compound showed that it had a value for pK_a of 7.98 ± 0.04 . This indicates that to develop the full colour the pH of the solution must be not less than 11. The absorption curve has two peaks, at 268.5 and 396 $m\mu$. A detailed examination of the reaction, as applied to the determination of glyceryl trinitrate, mannityl hexanitrate, sorbityl dinitrate, and pentaerythrityl tetranitrate showed that these compounds were quantitatively hydrolysed under the conditions chosen. The nitration of the *m*-xylenol gave an 82 per cent. yield of the nitro compound, and the recovery of the latter by steam distillation was 95 per cent. Thus

ABSTRACTS

an accurate analysis may be obtained by using a standard curve prepared from potassium nitrate. For tablets it is necessary to adjust the quantities according to the following table.

Substance	Wt. per tablet	a mg.	b ml.	c ml.	d ml.	e mg.	Solvent
Glyceryl trinitrate	0.00025—0.001	1.5—4	10	50.0	5.00	0.15—0.40	ether
Mannityl hexanitrate	0.005 —0.015	7.5—20	20	100.0	2.00	0.15—0.40	ether
Pentaerythrityl tetranitrate	0.030	15—40	20	100.0	1.00	0.15—0.40	acetone
Sorbityl dinitrate	0.002—0.010	6—15	10	50.0	2.00	0.25—0.60	ether

A weight of powdered tablets corresponding to *a* mg. of alkyl nitrate is extracted with *b* ml. of solvent for some minutes, and the solution is decanted through a filter paper, the extraction being continued with three more quantities of solvent, and the combined solution is made up to *c* ml. An aliquot of *d* ml. (corresponding to *e* mg. of alkyl nitrate) is transferred to a 250-ml. beaker, evaporated in a current of cold air, and treated with 1.0 ml. of a 2 per cent. solution of *m*-xylene in acetone and 10.0 ml. of 72 per cent. sulphuric acid. The beaker is covered and left for 30 minutes, after which time the reaction is stopped by adding 50 ml. of water. The mixture is then distilled into a cylinder containing 5 ml. of N/1 sodium hydroxide, the distillation being carried out slowly, taking in all about 5 to 8 minutes, and stopped when 15 ml. have distilled over. The product is made up to 50 ml. and the extinction determined at 447 m μ . The percentage of nitrate is determined from a standard curve obtained with potassium nitrate. If necessary, the ether solution may be washed with sodium sulphate solution before the hydrolysis to remove interfering substances. In the case of sorbityl dinitrate, white flecks may appear in the receiver and it is necessary to filter the solution before making up to volume. For ointments the method is similar, but it is necessary to agitate the mixture from time to time during the hydrolysis. Ointment bases such as soft paraffin or lanoline do not interfere.

G. M.

Bismuth, A New Reaction of. M. Jean. (*C.R. Acad. Sci., Paris*, 1948, 226, 85.) To 1 ml. of a slightly acid solution of bismuth nitrate (containing about 0.2 mg. of bismuth) is added 10 ml. of a solution containing 0.32 g. of rubanic acid and 6.5 g. of silicotungstic acid per l. A white turbidity is produced. On heating for 10 minutes in a water-bath a brown colour appears, proportional to the amount of bismuth. The optimum acidity for the reaction corresponds to 0.1N nitric acid; the limits being from 2N to pH3. A distinct reaction is obtained with 20 μ g. of bismuth at a dilution of 1 in 100,000. Interference is produced by metals which react with rubanic acid (copper, nickel, cobalt, ruthenium and platinum) or with silicotungstic acid (caesium), also by silver, mercury, zinc, cadmium and antimony. Chlorides weaken the reaction; acetates and tartrates prevent it. The reaction may be used for colorimetric determination.

G. M.

Calcium and Magnesium, in Solutions for Hypodermic and Intravenous Use, Determination of. R. Vigni. (*Ann. Chim. appl., Roma*, 1948, 38, 133.) Solutions of calcium and magnesium thiosulphates are used parenterally. To determine the amounts of the bases present in these solutions, the calcium is precipitated by adding a large excess of ammonium chloride, heating to boiling, making alkaline with ammonia and adding ammonium oxalate; the precipitate

is washed 8 or 10 times with ammoniacal water, heated to 40°C. for 2 hours to remove free ammonia, dissolved in dilute sulphuric acid and titrated with permanganate. The precipitate is free from magnesium, which will all be in the filtrate and washings. These are mixed, heated to 80°C. and a slight excess of the reagent recommended by Autanrieth added, which precipitates the magnesium as ammonio-magnesium phosphate. After 3 or 4 hours, the precipitate is washed by decantation with 2.5 per cent. ammonia, dried at 40°C. for 2 hours to remove excess of ammonia, and titrated with N/1 hydrochloric acid, using methyl orange as indicator, until an orange colour is obtained; 1 ml. of N/1 hydrochloric acid equals 0.01216 g. of magnesium. H. D.

Diamidines, Amperometric Microtitration of. J. B. Conn. (*Anal. Chem.*, 1948, 20, 585.) A number of diamidines were observed to give highly insoluble red alizarinsulphonates in neutral solutions, but attempts to use the reaction colorimetrically or gravimetrically failed: an amperometric titration procedure was successful. Alizarinsulphonic acid is polarographically reducible, the half-wave potential being -0.67 volt (against the saturated calomel electrode) and at -0.90 volt a steady diffusion current is reached. When a solution of diamidine salt in neutral buffer was titrated with a solution of sodium alizarinsulphonate a plot of the diffusion current at -0.90 volt against reagent volume could be resolved into two straight lines intersecting at a 1 to 1 equivalence point. The diamidines studied were (1) 4:4'-sulbamidocarboxyamidine disulphonate (sulbamidine), (2) 4:4'-oxydibenzamidine dihydrochloride (phenamidine), (3) 2,7-(trimethylenedioxy) dibenzamidine dihydrochloride (propamidine), and (4) 4:4'-(pentamethylenedioxy) dibenzamidine dihydrochloride (pentamidine). Graphs are given for the titration of these compounds, and also for their decomposition under sterilisation conditions. The overall reproducibility found was ± 0.5 per cent, the greatest spread of results being 1.5 per cent. for phenamidine.

R. E. S.

Digitoxin, Colorimetric Assay. A. T. Warren, F. O. Howland and L. W. Green (*J. Amer. Pharm. Ass. Sci. Ed.*, 1948, 37, 186.) In alkaline solution digitoxin gives a purple colour with sodium-8-naphthaquinone-4-sulphonate which changes to yellow on acidification. The yellow colour is stable and can be measured photometrically. Procedures are described for detecting digitoxin and for estimating it in powder and tablets. Interfering substances include lanatoside A and lanatoside C, which give strongly positive tests and lanatoside B and gitoxin, which give weakly positive tests; digitoxin gives a negative result. The intensity of the colour is increased by aldehydes, necessitating the use of aldehyde-free alcohol. The effect of lactose in inhibiting the formation of the purple colour was avoided in the assay of tablets by extracting with chloroform; no other excipient interfered. Results of the assay of 5 samples of digitoxin powder and 3 samples of tablets indicate the repeatability of the method, which should be useful for rapid routine analysis.

G. R. K.

Fatty Acids of Intermediate Chain Length, Estimation by Partition Chromatography. M. H. Peterson and M. J. Johnson. (*J. Biol. Chem.*, 1948, 174, 775.) The partition chromatograms used consisted of tubes packed with a coarse diatomaceous earth moistened with water or aqueous sulphuric acid as the non-mobile phase. As the developing solvent, thiophene-free benzene alone and mixed with Skellysolve B. and butanol-

an accurate analysis may be obtained by using a standard curve prepared from potassium nitrate. For tablets it is necessary to adjust the quantities according to the following table.

Substance	Wt. per tablet	<i>a</i> mg.	<i>b</i> ml.	<i>c</i> ml.	<i>d</i> ml.	<i>e</i> mg.	Solvent
Glyceryl trinitrate	0.00025—0.001	1.5—4	10	50.0	5.00	0.15—0.40	ether
Mannityl hexanitrate	0.005 —0.015	7.5—20	20	100.0	2.00	0.15—0.40	ether
Pentaerythrityl tetranitrate	0.030	15—40	20	100.0	1.00	0.15—0.40	acetone
Sorbityl dinitrate	0.002—0.010	6—15	10	50.0	2.00	0.25—0.60	ether

A weight of powdered tablets corresponding to *a* mg. of alkyl nitrate is extracted with *b* ml. of solvent for some minutes, and the solution is decanted through a filter paper, the extraction being continued with three more quantities of solvent, and the combined solution is made up to *c* ml. An aliquot of *d* ml. (corresponding to *e* mg. of alkyl nitrate) is transferred to a 250-ml. beaker, evaporated in a current of cold air, and treated with 1.0 ml. of a 2 per cent. solution of *m*-xylene in acetone and 10.0 ml. of 72 per cent. sulphuric acid. The beaker is covered and left for 30 minutes, after which time the reaction is stopped by adding 50 ml. of water. The mixture is then distilled into a cylinder containing 5 ml. of N/1 sodium hydroxide, the distillation being carried out slowly, taking in all about 5 to 8 minutes, and stopped when 15 ml. have distilled over. The product is made up to 50 ml. and the extinction determined at 447 m μ . The percentage of nitrate is determined from a standard curve obtained with potassium nitrate. If necessary, the ether solution may be washed with sodium sulphate solution before the hydrolysis to remove interfering substances. In the case of sorbityl dinitrate, white flecks may appear in the receiver and it is necessary to filter the solution before making up to volume. For ointments the method is similar, but it is necessary to agitate the mixture from time to time during the hydrolysis. Ointment bases such as soft paraffin or lanoline do not interfere.

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test for anthranols would appear to be desirable. Heating at 100°C. for one hour had no deleterious effect on the potency, but stabilised the anthraquinone content. Such a treatment might well be substituted for that of storage for one year.

G. M.

Morphine, Electrophotometric Determination of. Laura Nicolini. (*Ann. Pharm., Fr.*, 1947, 5, 528.) The official colorimetric method of the British Pharmacopœia for the determination of morphine is criticised. In the search for a reaction specific for morphine, which allows determination of the alkaloid without previous extraction, the author adapted the colorimetric method of Guarino. A solution, containing from 5 to 10 mg. of morphine, was placed in a 50 ml. flask and 10 ml. of N/10 hydrochloric acid added. The mixture was shaken, while 10 ml. of a 1 per cent. solution of iodic acid was added, followed after exactly 30 seconds, by 15 ml. of a saturated solution of ammonium carbonate. The mixture was allowed to stand for a short while with occasional shaking. The volume was adjusted with the solution of ammonium carbonate and several drops of a 0.2 per cent. solution of ferric chloride, acidified with N/5 hydrochloric acid, were carefully added drop by drop, till no further colour change took place. Excess of ferric chloride must not be added, since it causes precipitation of a hydrate or the iodide, which colours the violet solution yellow and would interfere with the electrophotometer reading. The assay was continued by Guarino's method, and colour intensity was estimated photometrically. Opium had first to be extracted by triturating with N/10 hydrochloric acid, and shaking for 20 minutes, before using this method. The photometer readings plotted against the content of morphine in mg., gave a straight line and obeyed Lambert-Beer's law.

L. H. P.

Senna Glycosides, Colorimetric Estimation of. By W. Kussmaul and B. Becker. (*Helv. chim. Acta.*, 1947, 30, 59.) A satisfactory and reproducible colour for the estimation of the glycosides sennoside A and B can be produced as follows: the aglycone is obtained by heating 10 ml. of a 0.5 to 1.0 per cent. alkaline solution of glycoside with 5 ml. of concentrated hydrochloric acid on a steam bath for about 15 minutes, when precipitation should be complete. After cooling, the precipitate is redissolved in concentrated sodium hydroxide, and the solution is extracted with 80 ml. of ether in a separating funnel, and then acidified with 50 per cent. sulphuric acid. The yellow ethereal layer is run off, any further precipitate in the aqueous layer is dissolved with more sodium hydroxide and the solution is shaken with 40 ml. of ether. The ether-fraction is next shaken with 3 or 4 quantities, each of 5 ml., of sodium bicarbonate solution. The mixed sodium bicarbonate solution is then extracted with 60 ml. of ether and acidified with 50 per cent. sulphuric acid. The aqueous layer is twice extracted with 20 ml. quantities of ether. Any deposit should be redissolved by the addition of alkali before shaking with ether. The mixed ether extracts are then filtered and the filtrate should be used within 10 hours. 5 ml. of the filtered solution is then extracted with 10 ml. of N/1 caustic soda. It is then treated with 0.2 ml. of 3 per cent. hydrogen peroxide and gently heated for about 4 minutes. A standard colorimeter curve is prepared by dissolving 30 mg. of pure sennoside B (dried in vacuo at 80°C.; equivalent to 18.75 of aglycone) in about 50 ml. of water with aid of a few drops of alkali. 10 ml. of this solution is hydrolysed with hydrochloric acid as above and 5 ml. of the resulting solution is oxidised as above. This solution is estimated in either a Zeiss-Pulfrich Photometer or a "Weka" Havemann photoelectric

ABSTRACTS

chloroform mixtures were used. Sulphuric acid (27 to 35N) was found to be a better solvent and a better non-mobile phase than water; using both these solvents quantitative separation of formic, acetic, propionic, *n*-butyric, caproic, caprylic, and capric acids is possible. Detailed procedure is given for the quantitative analysis of fatty acids in biological materials. Fatty acids, in known mixtures or fatty acids added to butter fat, were recovered with a maximum error of 8 per cent.

R. E. S.

Frangula Bark, Assay of. K. Erne. (*Svensk farm. Tidskr.* 1948, 52, 345, 377.) The method adopted for determining the biological activity of frangula bark was as follows: White mice were starved for 15 hours, and varying doses of the bark mixed with soft cheese, malt extract and water (8+8+1) were given. Observations were made after 4 hours. A positive reaction was shown by diarrhoea with copious yellowish-brown excretions. Each sample was tested on 3 groups of about 20 animals, each group receiving a different dose. The results were compared with those obtained with a sample which was taken as standard. Chemical tests were as follows. Total anthraquinones: 0.1 g. of the bark was refluxed with 30 ml. of M/2 sulphuric acid for half an hour. The mixture was then extracted in a separating funnel with successive 25-ml. quantities of ether until all the colour was extracted. The ether solution was filtered, and shaken out with 20-ml. quantities of M/2 sodium hydroxide. The alkaline solution was acidified with sulphuric acid and re-extracted with ether. The filtered ethereal extract was shaken with M/4 lead acetate until no more dark precipitate was formed, then extracted with M/2 sodium hydroxide, and the extract was made up to 50 ml. with the alkali. Ten ml. of this solution was diluted to 50 ml. with M/2 sodium hydroxide and, after 20 minutes, the extinction was determined at 530 m μ . The extinction coefficient for emodin is 3.88. Free anthraquinones: These were determined as above with the omission of the acid hydrolysis. Anthranols: 0.1 g. of the bark was refluxed with 10 ml. of M/2 sulphuric acid for 15 minutes, and the mixture extracted with 10-ml. quantities of ether until all the colour was extracted. The ether solution was dried with sodium sulphate, filtered, and passed through an alumina column (Brockmann) containing 5 g. of alumina. After developing the chromatogram with 50 ml. of dry ether, the yellow fluorescent zone was separated and treated with a few drops of a solution of 0.5 g. of selenious acid in concentrated sulphuric acid. A greenish black colour showed the presence of anthranols. The approximate percentage was determined by acid hydrolysis of the glycosides, extraction with benzene and petroleum ether, and chromatographing on calcium and magnesium carbonates, the anthranols being finally isolated and oxidised to anthraquinones which were determined photometrically. The results of the chemical and biological tests were not closely parallel, but it is considered that the chemical test is able to estimate the clinical value of the bark within about 20 per cent. The results obtained do not show any definite relation between bark thickness and physiological activity, although for a thickness below 0.7 mm. the activity was proportional to the thickness, and above this value the activity appeared to be constant, although for one sample of bark there was a marked peak at 2.5 mm. Thus the view that there is a maximum activity in bark from stems of 3 to 4 years old is not confirmed. There was in general no inequality between barks of the official thickness (1.5 mm.) and thicker ones. Anthranols may be present even in samples which have been heated at 100°C and then stored for one year. An official

parts; tragacanth, 40 parts. This is dusted on the concave face of a watch-glass which has previously been smeared with a thin layer of soft paraffin. The watch-glass is placed on a crucible containing the material to be tested. On heating the bottom of the crucible with a small flame, the presence of water is shown by a violet colour appearing on the watch-glass. The method will detect 2 mg. of water. The reaction is not given by organic liquids, and it may be used to detect water in pomades and other galenical preparations. G. M.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Potato Starch, Fractionation of. Part IV. Absorption Spectra and Colour Intensities of the Starch-Iodine Complexes. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 97.) Over the range 2400 to 7500 Å, the absorption spectra of the starch-iodine complexes of potato starch and its cold-water-soluble and hot-water-soluble fractions are very similar qualitatively to those of wheat starch fractions, the visible part of the spectrum (4300 to 7500 Å) being most sensitive to any changes in the starch. Depolymerisation by grinding lowers absorption only at the longer visible wave-lengths; at 5700 Å the effect, if any, is very small and disappears over the range 4300 to 5300 Å. Depolymerisation by grinding appears to consist in disruption of the unbranched parts of the amylopectin molecules and of the unbranched parts of the amylose molecules. The cold-water-soluble fractions of potato starch are richer in amylose and poorer in amylopectin than the hot-water-soluble fractions; in wheat starch fractions, the reverse is the case. The greater solubility of potato starch amylose in cold water is attributed to its lower molecular weight. The different behaviour of wheat starch is due either to the absorption by the amylose of the lipids present in the starch or to increased association between the amylose and amylopectin by means of hydrogen bonding, or to a combination of these factors. G. R. K.

INORGANIC

Clays, Effect of the Absorption of Sulphur on the Colloidal Properties of. A. Malquori. (*Ann. Chim. appl., Roma*, 1948, 38, 137.) The thixotropic index of clays, measured by Winkler's method, is increased by heating them with sulphur. The clay is thoroughly mixed with flowers of sulphur and heated in a tube for one hour at 130° to 140°C. For comparison the original clay is similarly treated without sulphur. The index increases up to a point with increased quantities of sulphur and then falls off, but the behaviour varies greatly with different clays. A sample of bentonite, which consisted of montmorillonite, increased up to 30 per cent. of sulphur, one of kaolin, consisting of kaolinite, up to 5 per cent., and one of Gavi clay (sericite) up to 15 per cent. The author discusses the relation of these different behaviours with the crystalline structures of the different types of clay, and connects it with the content of hygroscopic moisture.

H. D.

Hydrogen, Evolution from Ferrous Hydroxide. U. R. Evans and J. N. Wanklyn. (*Nature*, 1948, 162, 27.) Ferrous hydroxide, pure enough to be almost colourless, was precipitated by mixing ferrous chloride and potassium hydroxide solutions in an atmosphere of hydrogen. After precipitation the ferrous hydroxide and supernatant liquid were left in a flask connected to a manometer, but no hydrogen evolution was detected under these conditions even with ferrous chloride in excess. Further experiments

colorimeter and the readings are transferred to a graph which for both sennoside A and B is a straight line passing through zero.

A. D. O.

Sodium Diethyl- and Phenylethylbarbiturates, Acidimetric Titration of. H. Baggesgaard-Rasmussen and F. Reimers. (*Dansk Tidsskr. Farm.*, 1948, 22, 166.) Determination of the acidity exponents for diethylbarbituric acid and phenylethylbarbituric acid in aqueous alcohol gave the following figures. Diethylbarbituric acid, $pK_{8.96}$ (alcohol 50 per cent.); 9.54 (alcohol 75 per cent.); phenylethylbarbituric acid $pK_{8.61}$ (alcohol 50 per cent.), 9.20 (alcohol 75 per cent.). Since the barbituric acids are weaker in dilute alcohol than in water, the end point is sharper and there is no precipitation of the free acid during titration. The titration of the sodium salts is carried out as follows. 0.100 g. of sodium diethylbarbiturate or 0.1250 g. of sodium phenylethylbarbiturate is dissolved in a mixture of 10 ml. of alcohol (86 per cent. by weight) and 5 ml. of water, and titrated with aqueous N/10 hydrochloric acid to a green colour, using bromophenol blue as indicator.

G. M.

Sulphonamides, Microscopic Identification of. G. L. Keenan. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 202.) Optical crystallographic data and microchemical tests are described for sulphanilamide, sulphadiazine, sulphapyridine, sulphathiazole, sulphaguanidine and sulphamerazine, together with optical crystallographic data of certain of the complexes obtained in the microchemical tests. The tests described are based upon complexes formed with the following reagents: for sulphanilamide, aromatic aldehydes and silver nitrate; for sulphadiazine, gold bromide and hydrochloric acid; for sulphapyridine, gold chloride; for sulphathiazole, picrolonic acid; for sulphaguanidine, nitric acid; and for sulphamerazine, gold chloride and sodium bromide, and picrolonic acid.

G. R. K.

Sulphur in Organic Compounds, Microdetermination of. A. Steyermark, E. Bass and B. Littman. (*Anal. Chem.*, 1948, 20, 587.) Sulphur-containing organic compounds which gave low results by some micro-analytical methods were analysed satisfactorily by burning, following the Carius method, after which the resulting sulphate was titrated with barium chloride by the tetrahydroxyquinone indicator technique.

R. E. S.

Tragacanth, Powdered, Evaluation of. Report No. 1 of the Tragacanth Sub-Committee of the Analytical Methods Committee of the Society of Public Analysts. (*Analyst*, 1948, 73, 368.) This report deals with the measurement of the viscosity of mucilages made from the powdered gum. The falling sphere method and the U-tube method were rejected as being unsuitable or difficult to apply, and a method is recommended using a Redwood No. 1 viscometer. The principle of determining the concentration of the dry gum that would be required to produce a mucilage with a corrected efflux time of 100 sec. for 50 ml. of mucilage at 20°C. was adopted. Details of preparation of the mucilage, determination of viscosity and method of calculation are given. The moisture content is determined by drying about 1 g. of the powder in an open dish to constant weight in a steam-heated oven.

R. E. S.

Water, Detection of. P. Boymond. (*Pharm. Acta Helvet.*, 1948, 23, 207.) A powder which may be used for the detection of water is composed as follows: bromophenol blue, 5 parts; sodium carbonate, anhydrous, 15 parts; starch, 40

treated by tryptic digestion, and the heparin contents assayed by estimating the anticoagulating effects by means of the thrombin method of Jaques and Charles. The yield of heparin, expressed in mg. of "standard heparin," was 61.8 mg./kg. of liver, the tissue residue yielding 5.5 mg./kg., the precipitate 56.3 mg./kg., and the supernatant and dialysate nil. S. L. W.

Penicillin Standards. C. R. Bond. (*Analyst*, 1948, 73, 254.) The development and history of British standard penicillins is described, with details of a new working standard consisting of a crystalline sodium salt with an appreciably higher potency and containing only 0.4 per cent. of penicillin F in addition to penicillin G. A comparison of the composition of various penicillin standards is given, the determinations being made by Goodall and Levi's micro-chromatographic method. R. E. S.

BIOCHEMICAL ANALYSIS

Mercury in Organic Material, Determination of, by Polarographic Methods. G. Costa. (*Ann. Chim. appl., Roma*, 1948, 38, 157.) By the destruction of the organic matter by suitable means and conversion of the mercury to iodide the metal may be determined polarographically. For urine, pass a strong current of chlorine through 250 ml. at 80°C. for 1 hour; then pass a strong current of air in the cold for 1 hour, filter and concentrate on the water-bath to about 100 ml. Pass hydrogen sulphide through the liquid and, whether mercury is present or not, a brown precipitate is obtained. Set aside for 24 hours. Decant the clear liquid and centrifuge the remainder, washing the precipitate with a little water saturated with hydrogen sulphide. Then dissolve the precipitate in chlorine water, passing chlorine gas if necessary for a few minutes. The liquid remains turbid owing to the separation of sulphur. Filter into a graduated 20 ml. flask and make up to volume. This may be added to Schwartz's solution (potassium iodide, 4 g.; sodium acetate, 4.22 g.; gelatin (or, better, tylose) 0.2 g. in 100 ml. of water). Curves are given showing the results with different quantities of mercury and also the results of the presence of other heavy metals. The results are accurate within about 10 per cent. and 2 mg. of mercury per litre can be determined. H. D.

Œstrone, Equilin and Equilenin, Determination of, by Infra-red Spectrophotometry. J. Carol, J. C. Molitor and E. O. Haenni. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 173.) Mixtures of these ketosteroids were analysed by measuring the optical densities of solutions of their benzenesulphonyl esters in carbon disulphide at selected wave-lengths for each component in the infra-red region. The concentration of each component was calculated from graphs prepared by plotting optical density against concentration for each substance at the selected wave-lengths. The selected wave-lengths were 10.45 μ . at which equilenin shows maximum absorption, 10.88 μ , at which œstrone shows the maximum, and 10.96 μ , at which equilin shows the maximum. The benzenesulphonyl esters were chosen because they are readily prepared, show the greatest differences in absorption at the selected wave-lengths, and are sufficiently soluble in carbon disulphide. The method gave satisfactory results with 20 prepared samples, and with mixtures recovered from commercial oily solutions of natural œstrogens. G. R. K.

Penicillin, Assay by the Dilution Method. C. G. Pope. (*Analyst*, 1948, 73, 247.) A full description of this assay has already been published (Pope

following those of Schikorr also failed to yield hydrogen even when the reactants were heated to 100°C. Hydrogen was, however, evolved at room temperature with excess of ferrous sulphate if platinum chloride, colloidal platinum, nickel sulphate, nickel powder, copper powder or sodium sulphide was added. Manganese sulphate yielded no hydrogen under these conditions.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Antimalarial Agents and Their Action on the Glucose Metabolism of Plasmodia. P. B. Marshall. (*Brit. J. Pharmacol.*, 1948, 3, 1.) Using blood from chicks heavily infected with *Plasmodium gallinaceum*, the authors show that quinine and mepacrine exert inhibitory activity at several points in the glucose metabolism of plasmodia. Quinine inhibits hexokinase and phosphoglyceraldehyde dehydrogenase moderately, and possibly lactic dehydrogenase and pyruvate oxidation. Mepacrine inhibits hexokinase strongly, phosphoglyceraldehyde dehydrogenase moderately, and probably pyruvate oxidation. Further studies are necessary to find what proportion of the total inhibitory action takes place at the different points in the carbohydrate metabolism, and, indeed, whether the greater part of the inhibition does take place against the carbohydrate metabolism, or against other metabolic functions.

S. L. W.

Benzimidazole, The Folic Acid Activity and Antagonism of Two Structurally Related Derivatives of. P. C. Edwards, Dorothy Starling, A. M. Mattocks and H. E. Skipper. (*Science*, 1948, 107, 119.) Substances having an action like folic acid have previously been reported, and so have compounds which are antagonistic to folic acid. The authors have studied two benzimidazole derivatives for their folic acid activity or antagonism, because the benzimidazole nucleus closely resembles the purine nucleus and shows competitive action with amino-purines. The first compound examined was N-(4-[(2-benzimidazolyl)-methyl]-amino)-benzoyl]-glutamic acid in which the pterin nucleus (pyrimido-4:5-pyrazine) of pteroylglutamic acid had been substituted by the benzimidazole nucleus; it retained a certain degree of folic acid activity, as measured by the growth stimulating effects on *Streptococcus faecalis*. The other compound investigated was N-(4-[(2-benzimidazolyl)-methyl-amino]-benzenesulphonyl) glutamic acid, which had a sulphonyl group in place of the ketonic group of the first compound; the substance reversed the biological activity of folic acid on *S. faecalis* and became a metabolite antagonist. This reversal of activity is reminiscent of the essential metabolite antagonist theory for the sulphonamides, and throws some doubt on the specificity of the pteridin nucleus for the folic acid system.

L. H. P.

Heparin, a New Extraction Procedure for. O. Snellman, R. Jensen and B. Sylven. (*Nature*, 1948, 161, 639.) Solutions of potassium thiocyanate exert a pronounced power of extracting the mast cell granular substance from liver tissue fairly quickly and completely. Using M/1 potassium thiocyanate solution for 24 hours, the authors obtained about 90 per cent. extraction of the metachromatic material in ox liver. Histochemical examination of the tissue residues showed only insignificant amounts of metachromatic substances left after such extraction. After extraction the potassium thiocyanate was readily removed by dialysis, leaving the following fractions to be analysed: tissue residue, precipitate, supernatant and dialysate. These fractions were

of simple quaternary ammonium salts might be partly due to the presence of 2 such cationic groups at some optimum distance apart. The authors therefore prepared and tested for curare-like activity a number of simple *bis*-quaternary ammonium salts in which the nitrogen atoms were separated by polymethylene chains of different lengths. Among the salts prepared were polymethylene *bis*-trimethyl- and *bis*-triethyl-ammonium bromides, with chain-lengths of from 2 to 5 and 7 to 13 carbon atoms, and a smaller group of polymethylene *bis*-quinolinium and *bis*-strychninium dibromides; the drugs were tested on the phrenic nerve-diaphragm preparation of the rat. In the *bis*-trimethyl-ammonium series none of the compounds was more than 2/5 as active as tubocurarine chloride; on the other hand, using the rabbit head-drop test, the C_{10} member of this series was found to be about 3 times as potent as tubocurarine chloride, 0.08 mg./kg. being required to produce head-drop compared with 0.26 mg./kg. of *d*-tubocurarine chloride. Thus, the relative activities, and their variation within a homologous series, may be quite different in the rat diaphragm and the rabbit head-drop tests. As the augmentation of the contractions of the rat diaphragm produced by nearly all the *bis*-quaternary ammonium salts resembled superficially the effect of anticholinesterase drugs, the authors tested representative members on the cholinesterase of caudate nucleus (dog), with acetylcholine as substrate, and found that they all showed some inhibition of the enzyme at concentrations slightly lower than those needed to reduce the contractions of the rat diaphragm.

S. L. W.

Curare-like Action of Polymethylene *bis*-Quaternary Ammonium Salts. S. D. M. P a t o n and E. J. Z a i m i s. (*Nature*, 1948, 161, 718.) In a pharmacological study of a series of straight-chain aliphatic ω -*bis*-trimethyl-ammonium iodides, it was found that in the rabbit head-drop test for curare-like activity, the potency increased from the ethylene derivative to the octamethylene derivative, and the decamethylene derivative (C_{10}) was more potent still, 0.11 mg./kg. of the iodide being required to produce head-drop compared with 0.25 mg./kg. of *d*-tubocurarine chloride. The potency in relation to *d*-tubocurarine chloride varied however with the test object; thus, the approximate ratio of an effective dose of the C_8 derivative to an equipotent dose of *d*-tubocurarine chloride was, on the cat's tibialis, 1/3; on rabbit's head-drop, 3; on frog-nerve-sartorius preparation, 3; on rat's diaphragm preparation, 50 to 100. The curarine-like action was not antagonised by neostigmine in doses adequate to antagonise the effect of *d*-tubocurarine chloride either in the cat's tibialis or in the rabbit's head-drop test. Comparison of potency with *d*-tubocurarine chloride was complicated by the finding that while *d*-tubocurarine chloride is unaltered in potency when given after these *bis*-quaternary salts, the converse is not true; thus, following the injection of two-thirds head-drop dose, approximately twice as much of the C_{10} derivative was required to produce head-drop as was normally needed. The authors stress the importance of pharmacological testing of possible substitutes for *d*-tubocurarine chloride on more than one test object and suggest that before clinical application can be considered it is desirable to find some satisfactory antagonist to their effects.

S. L. W.

Streptomycin, Sulphetrone and Promin; Chemotherapeutic Action in Experimental Tuberculosis. G. B r o w n l e e and C. R. K e n n e d y. (*Brit. J. Pharmacol.*, 1948, 3, 37.) This report describes a comparison between the chemotherapeutic antituberculous activity of streptomycin, sulphetrone, promin, and combined streptomycin and sulphetrone. Four groups of 18 guinea-pigs, and one control group of 11, infected with a human virulent strain of *M.*

ABSTRACTS

and Stevens, *Bull. Health Org., L. of N.*, 1946, 12, 274.) and reference is made to essential details only. Growth curves are given from the practical results obtained, the purpose being to indicate the reason for the sharp end-point shown in this assay procedure. Figures are given of results obtained in comparative determinations of various standard penicillins and the advantages and disadvantages of the assay are tabulated. A method is outlined for the assay of penicillins G and K in commercial samples, depending on the fact that penicillins G and K give different values in terms of I.U./mg. when assayed against *Staphylococcus aureus*, while more nearly equal weights of each are adsorbed on charcoal.

R. E. S.

Penicillin, Investigation on the Iodimetric Method of Estimation of. A. M. Wild, (*J. Soc. chem. Ind., Lond.*, 1948, 67, 90.) The method depends upon measuring the amount of iodine absorbed by the penicilloates produced by alkaline hydrolysis (Alicino, *Industr. Engng. Chem., Anal. Edit.*, 1946, 18, 619; *Quart. J. Pharm. Pharmacol.*, 1947, 20, 59.) The results are shown to vary with temperature, concentration of potassium iodide in the iodine solution, pH, and the purity of the penicillin. The original suggestion that the effects of the impurities could be overcome by performing the blank titration immediately after addition of the iodine supposed that the impurities absorbed iodine immediately and that they were unaffected by alkali. Both suppositions are shown to be in error, and it seems doubtful whether the method can be used for the estimation of low-grade penicillin. For reasonably pure samples, the errors introduced are not large, and by strict control of conditions results are obtained which are more consistent and reliable than those from the usual bioassay. Using the modified procedure described, results have compared favourably with triplicate bioassays on the same samples. For samples of high penicillin G content, with potencies over 1000 units/mg., differences greater than 3 per cent. have been rare and the error progressively decreased as the samples tended to absolute purity. The repeatability of the method has been found to be of the order of ± 1.5 per cent.

G. R. K.

Penicillin, Microbiological Assay by the Turbidimetric Method. C. R. Bond and O. L. Davies. (*Analyst*, 1948, 73, 251.) The various factors affecting this assay method are discussed and a nutrient broth formula for *Staphylococcus aureus*, with details of the inoculum, is given. The following results of experimental work are quoted: small deviations in optimum temperature (37°C.) caused appreciable depreciation in growth; measurements over a period of $2\frac{1}{2}$ to $4\frac{1}{2}$ hours incubation showed a continuous increase in growth; the greatest rate of growth occurred at pH 7.5 while at pH 6.0 to pH 6.5 the growth rate was markedly retarded; the best bacteriostatic used to stop the growth of the test organism at the end of the incubation period was formalin. The advantages and disadvantages of the method are compared with those of the serial dilution and cylinder-plate methods. The standard errors found were: serial dilution, 10 per cent.; cylinder-plate, 4 per cent.; turbidimetric, 4 per cent.

R. E. S.

CHEMOTHERAPY

Curare-like Action of Polymethylene bis-Quaternary Ammonium Salts. R. B. Barlow and H. R. Ing. (*Nature*, 1948, 161, 718.) Tubocurarine chloride is a bis-tetrahydroisoquinoline alkaloid containing 2 quaternary nitrogen atoms, and its potency in blocking neuromuscular transmissions compared with that

less effective even than some of the common ionic agents. Certain of the polyvinyl alcohols were effective over a wide pH range. Methods of preparation of emulsions using dry powdered polyvinyl alcohol as well as viscous aqueous solutions are given.

R. E. S.

PHARMACOGNOSY

Datura metel, Effect of Colchicine Treatment on the Alkaloidal Content of. A. E. Beesley and G. E. Foster. (*Nature*, 1948, 161, 561.) In contrast to the results reported by J. M. Rowson (*Quart. J. Pharm. Pharmacol.*, 1945, 18, 175) that the treatment of seeds of *D. metel* and other solanaceous plants with a 0.4 per cent. aqueous solution of colchicine produced polyploid plants with a higher alkaloidal content than untreated seeds, a batch of *D. metel* seeds, similarly treated, produced plants which showed no evidence of polyploidy and no significant increase in alkaloidal content when compared with plants grown from a control group of seeds under conditions as far as practicable identical.

G. R. K.

Ergot and Preparations, Alkaloidal Content of. S. A. Schou, P. F. Jorgensen and V. G. Jensen. (*Dansk Tidsskr. Farm.*, 1948, 22, 161.) An examination was made of the alkaloidal content of 35 samples of ergot of varying geographical origin and harvests from 1938 to 1947. The content of ergometrine alkaloids was nil in 14 cases, while in 15 samples it was over 0.025 per cent., the maximum being 0.060 per cent. Five samples contained no ergotoxine alkaloids, 21 contained 0.100 per cent. or over, and the maximum was 0.217 per cent. For the new Danish Pharmacopœia it is recommended that the minimum limit for ergot should be 0.025 per cent. of ergometrine alkaloids and 0.100 per cent. of ergotoxine alkaloids: for the liquid extract the corresponding figures are 0.020 and 0.060 per cent. respectively. If 1 per cent. of ascorbic acid is added to the extract, and it is made from a drug containing the minimum proportion of alkaloids, the extract will still meet the requirements after storage for 6 months.

G. M.

Morphine Content of Poppy Capsules. H. Baggesgaard-Rasmussen and O. Lannig. (*Dansk Tidsskr. Farm.*, 1948, 22, 203.) A number of methods of assay were tried, the most satisfactory being a polarographic one, as follows. 2 g. of powdered capsules is moistened with 1 ml. of 1.2 M sodium carbonate solution and heated at 70°C. in a flask provided with a reflux condenser for 1 hour with 20 ml. of a mixture of butyl alcohol (7 volumes) and benzene (3 volumes). The mixture is then filtered through a sintered glass filter, the residue being pressed down and then washed with about 50 ml. of the mixed solvent. The extract is shaken out with 10 ml. of N/1 hydrochloric acid, and washed 3 times with 10 ml. of water. The aqueous extracts, which are practically colourless, are evaporated to 10 ml., treated with hydrochloric acid and potassium nitrate, then with excess of potassium hydroxide and the morphine is determined polarographically. The method was applied to experimental crops from seed of capsules tested the preceding year. The morphine content of the capsules of the second year (1947) was much higher than that of the preceding year, but this is probably due to the very different weather conditions. The distribution of morphine in the capsule was investigated by cutting the capsules into three equal parts by horizontal cuts,

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tuberculosis, were treated 22 days after infection with drugs for 168 days. One group received 0.5 per cent. of promin in the diet, one 2 per cent. of sulphetrone in the diet, one 10 mg. of streptomycin parenterally daily, and a fourth 2 per cent. of sulphetrone in the diet and 10 mg. of streptomycin parenterally daily; the control group of 11 animals remained untreated. On the basis of survival time, change in weight, response to tuberculin tests, macroscopic evidence of gross *tuberculosis post mortem* or microscopic examination, all presented a uniform picture of degrees of protection. The order of efficiency of the drugs was streptomycin with sulphetrone, streptomycin, sulphetrone, and promin. The protection given by the combined streptomycin and sulphetrone treatment was so marked as to be clearly synergistic. The authors express the opinion that the disease was progressive in all groups, though at a much suppressed rate in those groups where protection was greatest. Nevertheless, the experimental effects produced by the combined streptomycin and sulphetrone therapy are considered to justify a careful clinical evaluation in selected cases. S. L. W.

PHARMACY

GALENICAL PHARMACY

Strophanthus, Preliminary Report on the Extraction of. C. L. H u y c k. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 191.) Tinctures were prepared by percolation with alcohol (95 per cent.) and alcohol (65 per cent.) and assayed colorimetrically by a modification of the picric acid method of Knudson and Dresbach. The results given show there is little to choose between the solvents or the methods, except that the tinctures prepared with the weaker alcohol showed a somewhat greater loss of activity after 6 months and formed a precipitate.

G. R. K.

NOTES AND FORMULÆ

Polyvinyl Alcohol as an Emulsifying Agent. G. F. Biehn and M. L. Ernsberger. (*Ind. Engn. Chem.*, 1948, 40, 1449.) Commercial polyvinyl alcohols (partially or completely hydrolysed polyvinyl acetates) were examined for their interfacial tension and emulsifying properties. The most effective polyvinyl alcohols were found to be high-viscosity products hydrolysed to the extent of 75 to 80 per cent. At a concentration of 0.5 per cent. or higher (based on the total emulsion) these emulsifying agents compared favourably with other agents in giving emulsions of small droplet size, foaming was less, and where present the foam was unstable. Results on the stability of emulsions showed that the effectiveness of emulsifying agents generally varied greatly with different water-immiscible liquids. Comparisons with the polyvinyl alcohols showed that these agents and sodium dodecyl sulphate gave the most stable emulsions of trichloroethylene; for dibutyl phthalate, polyvinyl alcohols, sodium oleate and methyl cellulose were equally effective; for linseed oil, sodium alginate and sodium oleate were good, while two polyvinyl alcohols tested were fairly effective. The emulsion stabilities were measured by a method involving the measurement of the rate of separation of the internal phase under a constant centrifugal force. For emulsions containing sodium chloride, magnesium chloride or calcium chloride, the latter in concentrations corresponding to hard waters, a high viscosity 76 to 79 per cent. hydrolysed polyvinyl acetate gave stable emulsions, but other polyvinyl alcohols unexpectedly proved

para-Aminosalicylic Acid in Experimental Tuberculosis. W. T. McClosky, M. I. Smith and J. E. G. Frias. (*J. Pharmacol.*, 1948, 92, 447.) Tests in rats, guinea-pigs and rabbits showed a relatively low toxicity in all these animals, but chronic toxicity tests on guinea-pigs indicated a cumulative action. The compound is well absorbed from the gastro-intestinal tract and is well retained for several hours. Analysis of the urine of rabbits showed that during the 24 hours following ingestion, from 10 to 20 per cent. of the dose administered was excreted as the free compound and about 80 to 90 per cent. as the conjugated compound. It was found to have little therapeutic activity in rabbits infected with a bovine strain and in guinea-pigs infected with a human strain of tubercle bacilli. When given in combination with streptomycin the chemotherapeutic efficacy was no greater than the sum of effects from the two drugs; there was no evidence of potentiation as with the sulphones and streptomycin.

S. L. W.

Diisopropyl Fluorophosphate; Effect on Anoxic Survival. A. F. Freedman and H. E. Himwich. (*Science*, 1948, 108, 41.) It has been found that the use of diisopropyl fluorophosphate (D.F.P.) prolongs the survival period of medullary centres subjected to a complete arrest of circulation. If this increased resistance to anoxia observed in the isolated head should be found to apply also to the intact organism then it might be valuable in minimising the effects of anoxia. For this investigation the authors undertook a series of experiments under a variety of conditions, which included a comparison of the survival periods of animals previously injected with the drug with untreated controls, using (1) rats subjected to hypoxia, (2) the decapitated heads of new-born rats (length of gasping-time of head), (3) rats receiving excessive doses of pentobarbitone or of morphine. Only with morphine was there any suggestion of a beneficial effect from the previous use of the drug and on statistical analysis even this might be imputed to chance variation

S. L. W.

Dimercaprol (B.A.L.), Influence of, on the Toxicity and Therapeutic Activity of Mapharsen. N. Ercoli and W. Wilson. (*J. Pharmacol.*, 1948, 92, 121.) The influence of dimercaprol on the therapeutic activity of mapharsen was studied in relation to its toxicity in mice infected with *Trypanosoma equiperdum*. It was found that the curative or sterilising action of mapharsen is influenced much more readily by dimercaprol than the trypanocidal action, while the toxicity is the least affected. Thus, while the curative effect of mapharsen disappears with doses of dimercaprol as low as one-eighth to one-half the mapharsen dose, from 1.0 to 2.7 times more dimercaprol than mapharsen is required to interfere with toxic mapharsen doses. In other words the "therapeutic index" for the combined treatment is lower than for mapharsen alone, since the curative activity is more reduced than the toxicity by the same proportional dose of dimercaprol. In general, the higher the dose of the arsenical the more dimercaprol proportionately is required for inhibition. These findings preclude the possibility of combined chemotherapy with dimercaprol and mapharsen.

S. L. W.

Rutin, Effect on Anaphylactic and Histamine Shock. R. H. Wilson and F. DeEds. (*Science*, 1948, 107, 369.) The conclusions drawn by Raiman, Later and Necheles (*Science*, 1947, 106, 368) from their observation that rutin protects guinea-pigs from anaphylactic shock but not from histamine shock were that either histamine is not the direct cause of anaphylactic shock or rutin prevents the liberation of histamine. These conclusions are untenable in the light of results by other workers that rutin has a slight protective action

ABSTRACTS

the placenta and stigma being examined separately. Typical results were as follows:—

					Weight per cent. of total	Morphine (anhydrous) per cent. of total	Morphine per cent.
Upper part	16.5	0.31	11.8
Middle part	26.4	0.43	26.5
Lower part	34.1	0.52	41.0
Placenta	14.0	0.50	16.0
Stigma	9.0	0.24	4.7

G. M.

Opium from Greece. P. G. Kritikos. (*Pharm. Acta. Helvet.*, 1948, 23, 196.) A certain amount of opium is produced in Greece. Examination of a number of samples from different districts gave the following figures:—

	Sample						
	1	2	3	4	5	6	7
Moisture (100°C.) ...	6.38	3.48	4.03	3.68	6.01	3.54	5.78
Ash ...	2.90	3.35	2.97	2.65	2.08	4.98	3.60
Meconic acid ...	4.81	5.81	4.92	5.29	5.56	5.18	5.05
Narcotine ...	4.18	4.02	4.03	3.92	5.17	3.79	4.25
Codaine ...	1.50	1.27	1.27	1.17	1.22	1.25	1.57
Morphine ...	15.61	10.25	14.50	14.43	15.40	15.74	15.59

All the above percentages, other than the moisture content, are calculated on the dry material.

G. M.

PHARMACOLOGY AND THERAPEUTICS

Amidone, Pethidine and Morphine; Analgesic Effects in Human Subjects. E. N. Christensen and E. G. Gross. (*J. Amer. med. Ass.*, 1948, 137, 594.) A comparison in 11 volunteer subjects, employing the Wolff, Hardy and Goodell technique, showed amidone to be about three times as potent as morphine and many times more potent than pethidine when given subcutaneously, but that it lacked the sedative action of either of these drugs. Nausea and vomiting were not experienced by any of the subjects. With all three drugs the duration of analgesia following subcutaneous injection is much longer than that following intravenous injection. The simultaneous injection of a dose of 0.3 mg. of atropine decreased both the intensity and duration of the analgesic effects of both amidone and morphine, and the duration but not the intensity of pethidine. When atropine was given with any of the drugs administered intravenously the only consistent change was a shortening of duration of effect of amidone. The undesirable side-effects of morphine and pethidine, such as nausea and vomiting, were absent when atropine was given simultaneously. Sedation was more marked when atropine was injected with each analgesic. Simultaneous injection of 0.5 mg. of neostigmine increased both the intensity and duration of the analgesic action of all the drugs. From these studies, combined with the study of the clinical results in 69 patients to whom amidone combined with atropine was given as a pre-anæsthetic agent, the authors conclude that amidone is an unsatisfactory pre-medication agent, but is most useful as a post-operative analgesic and in pain from many other causes.

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against histamine, that death from histamine can be prevented by compounds closely related to flavonols and that scorbutic guinea-pigs have an increased sensitivity to histamine which is counteracted by a mixture of *d*-catechin isomers. Rutin affords protection against histamine shock in an indirect way and is not a true antihistaminic. The evidence that it protects against anaphylactic shock supports the theory that symptoms of anaphylaxis are produced by histamine.

G. R. K.

Thiouracil-treated Rats, Diffuse and Nodular Hyperplasia of the Thyroid Gland in. W. C. Kuzell, H. B. Tripi, G. M. Gardner and G. L. Laqueur. (*Science*, 1948, 107, 374.) 58 albino rats were fed on a basic diet containing 0.1 per cent. of thiouracil for periods of 34, 51, 120, 142 and 233 days. Comparison of the thyroid glands at the end of these periods with those from a control group of rats showed extreme hypertrophy in the glands from those animals fed on the thiouracil diet for a long time (the glands from male rats which had received thiouracil for 233 days averaged an increase in weight above normal of 489.3 per cent.). Histologically, the glands from animals removed from the thiouracil diet after 34 and 51 days showed no hyperplasia, whereas those from rats fed on the diet for 120 days or more were distinctly hyperplastic with areas of nodular hyperplasia; the number of nodules was related to the amount of thiouracil ingested. Since this experiment was part of a study designed to show the effect of thiouracil-induced hypothyroidism on experimental polyarthritis, all animals were inoculated at varying times with a broth culture of pleuropneumonia-like organisms, but there was no evidence that this had any effect on the thyroid glands.

G. R. K.

BACTERIOLOGY AND CLINICAL TESTS

Sodium *p*-Aminobenzoate, Bacteriostatic Properties of. R. Lecoq and J. Solomides. (*C.R. Acad. Sci., Paris*, 1948, 226, 846.) At dilutions of 1/130 to 1/250, sodium *p*-hydroxybenzoate has a bacteriostatic action, not only towards *Bact. coli* and the bacillus of Eberth, but also towards cultures of Gram-negative organisms—*B. dysenteriae* Shiga and *Vibrio Cholerae*. On the other hand it has no action on Staphylococci, Streptococci, *B. subtilis* and *B. diphtheriae*, even at 1/100. The development of *B. tuberculosis* (human and bovine) is inhibited even at a dilution of 1/1000. The action on moulds is variable: the growth of *Actinomyces griseus* is stopped at 1/250 while *Aspergillus niger* is resistant.

G. M.

Vitamin K, Antibacterial Analogues of, Effect on *Mycobacterium tuberculosis*. C. N. Ilan (Nature, 1948, 161, 1010.) During a search for chemically defined growth-factors for *M. tuberculosis*, it was decided to investigate vitamin K, which is present in many organisms and is probably of nutritional importance. A pigment, 'phthiocol' (2-methyl-3-hydroxy-1:4-naphthoquinone), isolated from a laboratory strain of tubercle bacillus, has vitamin K-like activity, and it has been suggested that phthiocol is derived from vitamin K during the extraction of the bacteria. It has long been known that *M. paratuberculosis*, when freshly isolated, will grow only on media containing extracts of other acid-fast bacteria, notably *M. phlei*, and it has been claimed that phthiocol and 2-methyl-1:4-naphthoquinone can replace *M. phlei*, but the stimulant action is not so marked, hence it does not follow that the *M. phlei* growth-factor and these compounds are the same. The author concludes that the original hypothesis that vitamin K-like compounds are necessary for the nutrition of *M. tuberculosis* has not been proved, but that there are certain indications that similar substances play some part in the metabolism of the organisms.

S. L. W.

PHARMACOPŒIAS AND FORMULARIES

THE BRITISH PHARMACOPŒIA, 1948

The Assay of Alkaloidal Salts

By D. VAN OS

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Chairman of the Netherlands Pharmacopœia Commission

IN the control of the purity of alkaloidal salts and similar compounds the question arises whether it is necessary to estimate the physiologically active base, or if it is sufficient to determine the other part of the compound molecule. In general, the estimation of the active base gives the best control of purity, but the methods are often laborious and troublesome. It is often difficult to find a good method for the estimation of the active substance, whereas a simple and quick method for the estimation of the other part of the molecule is readily available.

The British Pharmacopœia, 1948, selects the estimation of the active base, and it must be said that this standpoint is scientifically unassailable. For many substances the Pharmacopœia describes assays on this principle, including Amethocainæ Hydrochloridum, Amphetaminæ Sulphas, Cinchocainæ Hydrochloridum, Codeinæ Phosphas, Emetinæ Hydrochloridum, Homatropinæ Hydrochloridum, Mepacrinæ Hydrochloridum, Mepacrinæ Methanosulphonas, Morphinæ Hydrochloridum, Morphinæ Sulphas, Quinidinæ Sulphas, Quininæ Bisulphas, Quininæ Dihydrochloridum, Quininæ Hydrochloridum, Quininæ et Æthylis Carbonas, Quininæ Sulphas and Strychninæ Hydrochloridum. The principle of estimation of the active part of the molecule, however, is not introduced in the monographs on Apomorphinæ Hydrochloridum, Atropinæ Sulphas, Butacainæ Sulphas, Cocainæ Hydrochloridum, Diamorphinæ Hydrochloridum, Ephedrinæ Hydrochloridum, Hyoscinæ Hydrobromidum, Papaverinæ Hydrochloridum, Physostigminæ Salicylas, Pilocarpinæ Nitrates and Procainæ Hydrochloridum. Possibly the assay is not introduced in these monographs, because some of the alkaloidal salts have a sharp melting-point or a sufficiently narrow melting-range to guarantee the purity of the drug.

Four methods for the determination of the purity of such compounds may be discussed.

1. *The Method of the British Pharmacopœia, 1948.* In this the base is liberated by alkali from a solution of the salt and extracted by shaking several times with a suitable solvent, the solvent is evaporated, and the residue is dried and weighed or titrated. Such assays involve much work, and cannot be indicated as simple pharmacopœia methods.

2. *Chromatographic determination.* This method was described by Reimers, Gottlieb and Christensen.¹ I have used it for the salts of cinchocaine, cocaine, emetine, diethylmorphine physostigmine, pilocarpine, procaine, scopolamine and tetracaine, and find that it gives very good results. If the apparatus is kept ready for use and the worker has some experience, the chromatographic method requires less time and material than the extraction of the active base by a solvent. It is a condition for good results, that the aluminium oxide must be completely free from alkali and must give a good adsorption test.

3. *Titration of the acid.* This gives the correct percentage of the alkaloid

if it is correctly neutralised by its equivalent quantity of acid, and it is possible, by determining the acid part of the molecule, to get good control of the purity. The method is simply and quickly performed by titrating the alkaloidal salt solution with 0.1N sodium hydroxide, using phenolphthalein as indicator. If the alkaloid precipitates on adding sodium hydroxide solution, alcohol or chloroform is added to dissolve it. The method is used in the Netherlands Pharmacopœia for all the alkaloidal salts. If the alkaloid is not correctly neutralised in the salt, the titration will show a high percentage of alkaloid if there is too much acid, and a low percentage if there is too much base. It is possible to show that the alkaloidal salt has the correct composition by determination of the pH of an 0.1N solution. This can be calculated from the dissociation exponent of the base or estimated colorimetrically or electrometrically. Kolthoff² and Schoorl¹ have recorded the pH values of several alkaloidal salt solutions. Table I gives figures, determined in my laboratory, for the pH values of alkaloidal solutions of various concentrations

TABLE I

	0.1 N	0.01 N	1 per cent
	pH	pH	pH
Cinchocaine Hydrochloridum	4.75	—	—
Pilocarpine Hydrochloridum	4.58	4.73	—
Procaine Hydrochloridum	5.72	—	5.82
Ethylmorphine Hydrochloridum	4.58	—	5.10
Emetine Hydrochloridum	5.23	—	5.25
Hyoscyamine Hydrochloridum	—	—	4.93
Homatropine Hydrochloridum	5.79	—	—
Codeine Phosphas	4.24	—	—
Dihydrocodeinone Bitartras	3.32	—	—
Dihydro-oxycodone Hydrochloridum	—	6.12	—
Acetylmethyldihydrothebaine Hydrochloridum	—	5.85	—

The following experiment proves that the determination of the pH of an alkaloidal salt solution is a very good method for the control of purity and correct composition.

To a sample of atropine sulphate a small quantity of acid or of alkaloidal base was added to ascertain if the pH change of the solution was practically measurable. Both substances caused a considerable change in the pH of the solution. Figures are given in Tables II and III.

TABLE II

THE EFFECT ON pH OF THE ADDITION OF ACID TO A SOLUTION OF ATROPINE SULPHATE

Concentration	pH	pH after addition of 0.05 ml of N/1 acid	Difference	pH after addition of 0.1 ml of N/1 acid	Difference
712 mg. of atropine sulphate in 20 ml. of water	5.10	4.20	-0.90	3.78	-1.32
	5.09	4.18	-0.91	3.76	-1.33
	5.10	4.18	-0.92	3.76	-1.35

The conclusion is that the pH determination is a very good test for the correct neutralisation of the base by the acid.

4. *Double Titration* This method⁴ consists of a titration of the acid and basic parts of the molecule. A milli-equivalent of the alkaloidal salt is dissolved in 5 ml. of water, this solution is mixed with 15 ml. of alcohol (96 per cent.) and titrated with 0.1 N sodium hydroxide with phenolphthalein

ASSAY OF ALKALOIDAL SALTS

as indicator. Theoretically 10 ml. should be required. Then bromothymol blue is added and the liberated base is titrated by 0.1 N acid, of which also 10 ml. should be required. In the first titration, the first pink colour gives the end of the titration. Strong bases (atropine, emetine, etc.) which are

TABLE III

THE EFFECT ON pH OF THE ADDITION OF ATROPINE BASE TO A SOLUTION OF ATROPINE SULPHATE

Concentration	pH	pH after addition of 10 mg. of base	Difference
712 mg. atropine sulphate in 20 ml. of water	4.90	7.90	+3.00
	4.91	7.94	+3.03
	4.93	7.94	+3.03

alkaline to phenolphthalein must be extracted by means of an organic solvent such as chloroform. For the second titration, the alkaloidal base must be dissolved in as little alcohol as possible. If the alkaloidal base is extracted and dissolved in chloroform, it is possible to : (a) add an excess of acid and extract the base in the aqueous layer, after which the free acid is titrated with sodium hydroxide; (b) evaporate the solution and dry the alkaloid (if not volatile) dissolve it in an excess of 0.1N acid and titrate with 0.1N sodium hydroxide. The double titration can also be done with microburettes, and only a small quantity of material is then required. The method gives good control of the correct neutralisation of the alkaloid by the acid and also of the correct composition of the drug and directly the percentage of the active principle.

The conclusions of my experience are: 1. The direct determination of the active principle is scientifically unassailable but often requires complicated assays; 2. The direct determination by extraction with a solvent can be replaced by a chromatographic determination if a pure aluminium oxide is available; 3. The active principle can be determined indirectly by a simple titration of the acid part of the molecule and the determination of the pH of a dilute solution of the alkaloidal salt; 4. The same result can be obtained by the double titration of the acid part and alkaloid.

Methods (3) and (4) can be recommended for the control of purity and correct composition for all alkaloidal salts mentioned in the Pharmacopœia where there is no assay prescribed.

The author wishes to express his sincere thanks to Mr. J. S. Faber, Conservator of the Pharmaceutical Laboratory, University of Groningen, for his help in this work.

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BOOK REVIEWS

THE CHEMISTRY OF THE POLYSACCHARIDES by R. J. McIlroy. Pp. 116 and Index. E. Arnold & Co., London, 1948, 10s. 6d.

Though the nature of the simple sugar units present in polysaccharides had previously been recognised from examination of hydrolytic products, it is only in the last sixteen years that techniques have been developed which have led to our present conceptions of the chemistry and structure of a wide range of polysaccharides and their derivatives, of both animal and vegetable origin. It was in 1932 that Haworth and Machemer showed that with methyl alcoholic hydrogen chloride, the terminal unit of fully methylated polysaccharides was obtained as a methylated methyl glycoside and that by refined distillation methods it was then possible to separate and determine the proportion of the terminal unit. Haworth's "cnd group assay method" has since been widely applied. It has led to our present knowledge of the chemistry of starch, including both the water-soluble component, amylose, and the water-insoluble component, amylopectin, as well as to confirmation of the amylose-nature of the 'synthetic' starch obtained by Hanes in 1940 by submission of glucose-1-phosphate to the action of a purified phosphorylase obtained from potato tuber juice. It has led also to modern conceptions of the chemical nature of cellulose, of the pentosans, of glycogen, of mucilages and gums, of carbohydrate products of bacterial metabolism, and of the immuno-polysaccharides. The importance on the one hand of the carbohydrate sulphuric ester, heparin, the natural blood anti-coagulant stored in the liver and heart, and on the other hand of the antigenic polysaccharides derived *inter alia* from pneumococci, tubercle bacilli, and the relation of carbohydrate haptens to the polyuronides (gums), are such as to emphasise medical aspects of the need for development of knowledge of polysaccharide chemistry. The advancing front of chemical study of polysaccharides of diverse origin is now such that there has been real need for a concise readable resumé of modern developments in the field. This need is admirably met by the book under review. The structure of relevant monosaccharides is discussed briefly as an introduction to an account of modern methods of determination of polysaccharide structure. Chapters follow on starch and cellulose, glycogen, levans, galactose-, mannose-, amylase- and immuno-polysaccharides, and the polyuronides including hemicellulose, pectin, gums, mucilages and alginic acid. Part II of the book describes the derivatives of monosaccharides of importance in polysaccharide investigations, methods for estimation of carbohydrates, the utilisation of carbohydrates in animal and plant metabolism, the role of carbohydrates in immunology, and the identification of sugars and sugar acids. An appendix completes the survey of available literature to September, 1947. A comprehensive list of references at the end of each chapter permits extensive condensation of the text without in any way detracting from the clarity of the discussions. The book can be commended to all those who, unable to keep in touch with an ever expanding literature of a developing subject, yet desire, or have need, of an up-to-date knowledge of the methods of investigation, the problems arising and the results achieved in this important branch of chemistry. F. HARTLEY.

THE BASIS OF CHEMOTHERAPY by T. S. Work and E. Work. Pp. 435 + XX. Figs. 42. Oliver and Boyd, Ltd., Edinburgh, 1948, 26s.

It is refreshing to receive for review a new work founded upon an original idea. The book was designed to give young research workers a broader and sounder basis for the study of chemotherapy. It is my belief

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that this aim will be achieved, but I would not call the work a text-book: rather, it is a scientific monograph. After a short historical survey, the succeeding chapters, Cell Metabolism; Essential Metabolites; Enzyme Inhibition; Drug Antagonism; Drug Resistance, lead logically to the final consideration of the relation of structure and activity. In each case the known facts are discussed in relation to their effect on the problems encountered in chemotherapy. The whole is very stimulating to thought, and, as one reads, research problems frequently suggest themselves. The authors themselves admit that quicker results in chemotherapeutic research may yet be obtained by the older empirical method than by following suggestions based upon knowledge of reactions taking place in living cells. For some time it will be more important to gain a more accurate knowledge of such reactions than to apply them to chemotherapy. It may be that the chemical reactions of living cells may never provide the basis for chemotherapeutical design because of the similarity of one cell with another. The cells of the host and those of the invading organism may be too much alike in chemical sensitivities to be differentiated in this way. However, no matter what the ultimate result may be, the worker in the field of chemotherapy cannot help but benefit by a fuller knowledge of those subjects covered by this book. There is one little grouse that I would like to make. This book mentions a large number of medicinal chemicals, and in most cases ignores the Pharmacopœial names for them. Do all biochemists speak of thiamin rather than aneurine? It would be better to include the official names, even though they be given in brackets. The book is remarkably free from errors, and is exceedingly well documented (the bibliography covers 54 pages), the references including work done in 1946. Perhaps it would be fair to point out that the group of workers mentioned on page 334 did not synthesise for the first time all the compounds treated in Table 34. The book affords the worker in closely related fields the opportunity of obtaining, in a summarised and palatable form, the results of research on the biochemistry of the living cell, and I have every pleasure in recommending it highly as accurate, authoritative and well written.

W. H. LINNELL.

BOOKS RECEIVED

PRACTICAL METHODS IN BIOCHEMISTRY by F. C. Koch and M. E. Hanke. Pp. 420, Ballière, Tindall and Cox. 1948, 5th ed., 16s. 6d.

THE STUFF WE'RE MADE OF by W. O. Kermack and P. Eggleton. Pp. 350 and Index. Edward Arnold and Co., London, 1948, 2nd ed., 10s. 6d.

THE PRESENTATION OF TECHNICAL INFORMATION by Reginald O. Kapp. Pp. 140 and Index. Constable and Co., Ltd., London. 1948, 6s

A MANUAL OF PHARMACOLOGY by T. Sollman. Pp. 1132. W. B. Saunders Company, London. 1948, 2nd ed., 57s. 6d.

AMERICAN PHARMACY, edited by R. A. Lyman. Vol. 1. Pp. 522, 1948. 2nd ed. Vol. 2. Pp. 328, 1947. J. B. Lippincott Company, Philadelphia.

PHARMACOLOGY, THERAPEUTICS AND PRESCRIPTION WRITING by W. A. Bastedo. Pp. 840. W. B. Saunders Company, London, 1947, 5th ed., 42s.

LETTERS TO THE EDITOR

An Identity Test for Pheniodol

SIR,—During recent work in our laboratories an identification test suitable for routine analysis was required for pheniodol, α -phenyl- β -(4-hydroxy-3:5-diiodophenyl)-propionic acid, but no assistance in this matter was obtained by a survey of the literature. Accordingly, we developed the following test which has proved satisfactory in the hands of several independent workers.

Dissolve 0.5 g. in 15 ml. of 10 per cent. sodium hydroxide solution, warming if necessary. Add 1 g. of zinc dust and boil the mixture under a reflux condenser for 20 minutes. Cool, filter, add excess of dilute hydrochloric acid and collect the α -phenyl- β -(4-hydroxyphenyl)-propionic acid. Wash with water and recrystallise the product from alcohol-water mixture. The crystals, after drying at 100°C., melt at 180° to 181°C. The filtrate, from the acid after separation from the original reaction mixture, affords reactions characteristic of iodides.

It is felt that this test may be of interest to your readers.

Wellcome Chemical Works, Dartford.
October 21, 1948

G. E. FOSTER.
W. D. WILLIAMS.

Silicotungstic Acid

SIR,—In the discussion at the British Pharmaceutical Conference on our paper entitled "The Chemical Determination of Aneurine in Tablets and Ampoule Solutions" (see *Quart. J. Pharm. Pharmacol.*, 1948, 21, 370, 423) a question was asked as to the composition of the silicotungstic acid used in our experiments. The composition may be of importance, as is reputed to be the case in the method of the Association of Official Agricultural Chemists for the determination of nicotine (*Methods of Analysis*, 6th Ed., p.74). We obtained the silicotungstic acid for our work from only one source and the suppliers have advised us that the composition varies slightly but approximates closely to $H_4SiW_{12}O_{40} \cdot 24H_2O$. This corresponds to the formula quoted by the A.O.A.C. for the nicotine determination, $4H_2O \cdot SiO_3 \cdot 12WO_3 \cdot 22H_2O$.

Roche Products, Ltd., Welwyn Garden City.
November 3, 1948.

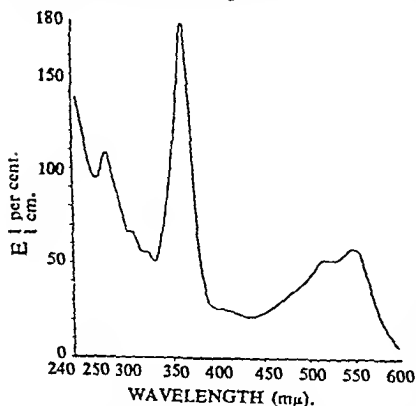
D. C. M. ADAMSON.
F. P. HANDISYDE.

The Isolation of the Crystalline Anti-Pernicious Anæmia Factor from Liver

SIR,—Work on the anti-pernicious anæmia (A-P-A) factor present in liver, in progress in these Laboratories for some years, has led to the isolation of a red crystalline compound from anahæmin, probably identical with the vitamin B_{12} of Rickes *et al*¹ and with the crystalline A-P-A factor of Smith and Parker². The methods of purification employed by us, however, differ in certain respects from those hitherto revealed. Thus the observation that the A-P-A factor is extracted by *n*-butanol from its aqueous solutions in the presence of fairly high concentrations of ammonium sulphate³ enabled us to effect enrichment of the fractions at various stages of the process. Chromatography was reserved only for the final purification. Columns of bentonite or aluminium silicate³ were used, and, under carefully controlled conditions, proved eminently satisfactory by giving rise to the formation of sharply defined red bands. These, after dissection and elution, gave material which readily crystallised in small red needles from aqueous acetone.

Our crystalline product, after drying *in vacuo*, contained 4.0 per cent. of cobalt, a figure identical with that reported by Smith⁴. In aqueous solutions

it shows characteristic light absorption (see Fig.). A main band appears in the visible region of the spectrum with a maximum at $500\text{ m}\mu$ and a "shoulder" at approximately $520\text{ m}\mu$, whilst two distinct maxima occur in the ultra-violet, one at $361\text{ m}\mu$ and the other at $278\text{ m}\mu$, with inflections at $322\text{ m}\mu$ and $304\text{ m}\mu$.



Several batches of the crystals have been hydrolysed with 20 per cent. hydrochloric acid in sealed tubes at $100^{\circ}\text{C}.$, and the hydrolysates examined by uni-dimensional paper-strip partition chromatography, using the technique described by Consden *et al.*⁵. The chromatograms obtained have consistently revealed the presence of only one substance reacting with ninhydrin.

Using aqueous *isobutyric* acid as the solvent, a pronounced purple spot appears on the paper at a point approximately mid-way between the positions occupied by the α -amino-acids valine and *norvaline*. With aqueous phenol or *n*-butanol as solvents, however, the colour of the spot is greatly diminished in intensity although its position with respect to the two amino-acids remains substantially unchanged. Experiments to detect the presence of purines⁶ in A-P-A hydrolysates have not, so far, been successful. Vigorous hydrolysis of the A-P-A factor with 20 per cent. hydrochloric acid under reflux for 8 hours failed to rupture the cobalt-containing complex present in the molecule. Removal of a hydrophilic fragment undoubtedly takes place as the product is readily and quantitatively extracted from the diluted hydrolysate with *n*-butanol (cf. Smith⁷). The product is an almost black, amorphous solid which is acidic in character as it is soluble in dilute alkalis and is reprecipitated unchanged on neutralisation. This "acid" is insoluble in ether, chloroform and acetone, but is rendered soluble in these solvents by the addition of a trace of hydrochloric acid. Its light absorption (measured in dioxan) shows a maximum at $557.5\text{ m}\mu$ with a "shoulder" at $530\text{ m}\mu$, and is thus similar (in the visible part of the spectrum) to the absorption of the A-P-A factor itself. The retention of a cobalt co-ordination complex in the molecule after the somewhat drastic acid hydrolysis is surprising, but is paralleled by the behaviour of certain metal porphyrins. Supplies of the methyl ester of the "acid" are now being accumulated to enable us to undertake its more detailed study.

The authors thank Dr. R. E. Stuckey for the analytical and absorption data and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

Research Department,

The British Drug Houses, Ltd., London, N.1.

November 24, 1948.

B. ELLIS.

V. PETROW.

G. F. SNOOK.

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SCIENTIFIC MEETING

DRUG ACTION, IONS AND NEUTRAL MOLECULES

by ADRIEN ALBERT, D.Sc. Ph.D. B.Sc., F.R.I.C.

Summary of a paper read at the Norwood Branch of the Royal Institute of Chemistry, at the Norwood Technical Institute on December 6.

MANY drugs undergo ionisation and a number do so to different degrees at different pH values. The most significant changes in ionisation occur around the pH value at which the drug is 50 per cent. ionised, i.e., the pK_A value. Ions and molecules behave differently in their effects on drug action, especially in relation to chemical reactivity, adsorption and the penetration of membranes. An example of the first difference is shown by aniline, where the molecule is nitrated mainly in the *para* position, but the ion is nitrated in the *meta* position. Again the mono-anion of ascorbic acid is easily autoxidised, whereas the di-anion and the molecule are both quite stable. A distinction is made between general and specific adsorption. In the former, the drug is rejected by water because of a relative lack of hydrophilic groups and becomes adsorbed on any surface which offers itself. In such cases the molecule is usually more highly adsorbed than the ion, because the latter is hydrated at one end. In specific adsorption the drug, which may have many hydrophilic groups, is specifically attracted to cellular receptor-groups by reason of having a complementary structure. This often takes the form of the drug being kationic and the receptor anionic, or *vice versa*. In such a case, only the ion can be adsorbed. Generally, for any one substance, the molecule penetrates much faster than the ion. The principal barrier to the penetration of an ion is the strong attraction between its charge and the oppositely charged groups on the cell membrane. However, if ions are provided with lipophilic groups, penetration is considerably improved.

In view of these marked physico-chemical distinctions between ions and molecules, it is not surprising to find that some drugs (such as the acridine antibacterials) have ions that are many times as active as the neutral molecules. Other drugs are known (e.g. benzoic acid) where the activity is proportional to the amount of non-ionised material present. Information of this kind can be gathered from reasonably simple experiments, in which firstly a given drug is examined biologically over a range of pH values and then a series of related substances, differing widely in pK_A values, is examined at the physiological pH value (pH 7).

Such information is of considerable practical use. Modern knowledge of inductive constants enables the pK_A of a substance to be varied at will by the insertion of appropriate groups, so that ionisation can be either increased or repressed at a given pH value. In some classes of drugs, such as the sulphonamides, a more complex picture is presented, where the maximum activity is obtained when the drug is half ionised. The usual interpretation of this is that the neutral molecule is required in order to penetrate into the cell, but that the ion is regenerated within the cell (according to the law of mass action) and is, of the two, the more biologically active.

NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Benecol* is an extract of mammalian intestinal mucosa, standardised on its content of erepsin, the mixture of proteolytic enzymes secreted by the intestine and responsible for the final stages of protein degradation to amino-acids. It is claimed that administration of this extract compensates for the poor intestinal functioning commonly associated with arthritis. It is administered in capsules, which pass unchanged through the stomach and disintegrate high in the small intestine, the dosage being 1 capsule 3 times daily, increased to 3 capsules 3 times daily, between meals. The treatment is continued for several months. Benecol is indicated in rheumatoid arthritis and in all types of rheumatic disease and is issued in boxes containing 100 capsules. S. L. W.

Diamidin* is a proprietary brand of 4 : 4'-diaminodiphenylsulphone disodium formaldehyde sulphonylate, and is claimed to have given promising results in the oral treatment of leprosy. The dose is 0.33 g., increased to 1 g., daily, at least 6 months' treatment being required before clinical effectiveness can be evaluated. A combination of promin intravenously and diamidin orally has been suggested as a logical procedure. The treatment may give rise to various toxic reactions, and the blood picture must be watched carefully; it is common for patients to develop an anaemia during the initial stages of treatment. Enteric-coated tablets of diamidin, each containing 0.33 g., are supplied in bottles of 100 and 1,000. S. L. W.

Nicorbin* is a combination of aneurine hydrochloride, 1 mg., ascorbic acid, 25 mg., and nicotinic acid, 10 mg., in tablet form. 3 tablets supply the approximate daily adult requirements for these vitamins. The tablets are indicated in minor deficiency states manifested by debility and fatigue and for routine prophylaxis during pregnancy and lactation. Nicorbin is issued in bottles of 25 and 100 tablets. S. L. W.

Pectalin* is a suspension of colloidal kaolin, 40 gr., with pectin, 8 gr., in a carminative vehicle. It is administered in a dose of 1 or 2 tablespoonfuls four-hourly to adults, in the treatment of bacillary dysentery, diarrhoea and food poisoning; it is also of value in the treatment of the summer diarrhoea of infants. It is issued in 8 fl. oz. bottles. S. L. W.

Somalcos* is an aqueous solution containing 10 per cent. of sodium camphor-sulphonate for injection. It possesses the stimulating action of camphor on respiration and circulation, but is more rapidly absorbed and is practically non-toxic. It is employed by subcutaneous, intramuscular or intravenous injection, in a dose of 1 or 2 ml. as a restorative in collapse, especially in pneumonia and acute fevers. In cases of poisoning by carbon monoxide, gas or narcotics, as much as 5 to 15 ml. may be given intravenously. It is supplied in boxes of 6 or 12 ampoules, each ampoule containing 0.1 g. in 1 ml., and in vials of 15 ml. S. L. W.

prevented this conversion occurring. The former suggestion appeared to be contradicted by the observations of Chaikoff and his collaborators² on the formation of radio-diiodotyrosine and radio-thyroxine in isolated slices of thyroid, for they found that thiouracil, thiourea and the sulphonamides inhibited this formation, while in no way hindering the uptake of iodide by the slices from the fluid medium in which they lay. However, this was not convincing proof of the mode of action of anti-thyroid drugs in the intact animal, for it could be argued that the transference of iodide from an artificial fluid into the thyroid cell was a very different process from the transference of iodide from capillary to cell in the living animal. Thus while it was clear enough from Chaikoff's work that the anti-thyroid drugs could prevent the formation of diiodotyrosine and thyroxine, it remained possible that in the intact animal they also prevented the thyroid from collecting inorganic iodide from the bloodstream.

However, it presently became apparent from the work of McGinty and Sharp³, Astwood⁴ and Vanderlaan and Bissell⁵ that the iodine content of the thyroid, under the influence of *anti-thyroid* drugs, could still vary with the iodine content of the diet. Astwood⁴ showed that when rats, who were receiving adequate amounts of thiouracil in the diet, were given a single dose of iodide by injection, the total iodine content of the thyroid rose sharply as soon as 1 hour after the injection. Vanderlaan and Bissell showed that the iodine thus taken up disappears again from the thyroid a short time later. Since it is unlikely that iodide could have become bound to protein and left the gland in so short a time it seemed probable that the temporary increase in thyroid iodine was all in the inorganic form. The three groups of workers also provided direct evidence that the iodine was, in fact, not bound to protein, and was therefore probably inorganic. Further confirmation came from the detailed studies of Vanderlaan and Vanderlaan⁶ and of Taurog, Chaikoff and Feller⁷, who showed, by a variety of methods, that the iodine which enters the thyroids of rats treated with propylthiouracil is indisputably in the form of inorganic iodide. They showed that under these conditions the non-hyperplastic thyroid (of rats given a single injection of propylthiouracil 1 hour previously) concentrated iodide to about 25 times the concentration present in plasma. The hyperplastic thyroid of rats chronically treated with propylthiouracil concentrated iodide to about 250 times the concentration present in plasma unless the latter was inordinately high.

This work shows that the thiouracil compounds do not prevent the thyroid from concentrating iodide to a high degree. But though iodide can enter the gland, it cannot become linked to protein. Therefore it cannot be retained in the gland. Thus Vanderlaan and Vanderlaan⁶ showed that the normal rat thyroid can retain 20 times as large a proportion of a dose of radioactive iodine as can the thyroid of a rat which has had a single injection of propylthiouracil. The early observation of Astwood and Bissell⁵, that treatment with thiouracil lowers the iodine

content of the thyroid, can now be explained. 'Thiouracil does not stop iodine getting into the thyroid; it prevents it from being retained there.'

Vanderlaan and Vanderlaan⁶ also showed that thiocyanate prevented the concentration of iodide by the thyroids of rats treated with propyl-thiouracil and, moreover, caused any iodide which was present to be discharged. When thiocyanate is given, the concentration of iodide in the thyroid becomes approximately equal to that in the blood. Thiocyanates can cause goitre, by preventing the thyroid from concentrating iodide. But if there is an excess of iodide in the diet—and hence in the blood—enough iodide can enter the gland even if the concentrating mechanism is out of action. Hence thiocyanates do not cause goitre in the presence of an excess of dietary iodide (Astwood⁸).

The work of Vanderlaan and Vanderlaan⁶, and others, has thus shown very clearly the existence of two separate mechanisms in the thyroid: the mechanism which concentrates iodide from the blood, which is inhibited by thiocyanate; and the mechanism which combines iodide with protein, and stores it in the gland, which is inhibited by the thiouracil derivatives. This new conception has been made use of by Stanley and Astwood⁹ in a study of the uptake and discharge of radioactive iodide by the thyroid in normal and thyrotoxic human subjects. Under the influence of a thiouracil derivative the proportion of a tracer dose of iodide taken up by the thyroid is reduced, and the peak of the uptake curve is lower and reached much earlier. The work of Vanderlaan and Vanderlaan⁶ makes it clear that what is being observed under these conditions is the uptake of inorganic iodide by the thyroid, and its rapid subsequent dilution by non-radioactive iodide, since the thyroid (under the influence of thiouracil) is incapable of binding it to protein. The ability of thiocyanates to discharge iodide from the thyroid made it possible to estimate the proportion of the radioactive atoms which are built up into thyroid hormone under any given conditions. Thus if a thyrotoxic patient, under treatment with a thiouracil derivative, is given a tracer dose of radioactive iodide, and then (after an interval) an adequate dose of thiocyanate, the amount of radioactivity remaining in the thyroid region is an index of the proportion of the administered iodide which has been converted into thyroxine and is bound to protein. In this way an estimate is obtained of the efficacy of the anti-thyroid drug being administered. The practical value of this method is excellent evidence that the conclusions of Vanderlaan and Vanderlaan⁶ apply also to the human thyroid, whether normal or thyrotoxic.

THE EFFECT OF THIOURACIL UPON THE COMBINATION OF IODINE WITH PROTEIN

It may be assumed that iodine enters the thyroid in the form of inorganic iodide. But the normal thyroid contains little iodine in this inorganic form. It follows that very soon after its entry into the gland the iodide must enter into combination with protein.

The exact form which this iodine-protein complex takes can at present only be surmised from indirect evidence. Taurog and Chaikoff¹⁰ have

shown that from the blood of normal animals there can be extracted—without the use of any destructive measures—a substance whose properties correspond with those of thyroxine. The same method of extraction failed to remove a similar substance from the thyroid gland. Since this suggests that there is no free thyroxine in the gland, it may be supposed that the tyrosine which becomes iodinated to form thyroxine is already part of a protein molecule when the process takes place. Release of free thyroxine into the circulation would take place when this protein is broken down by enzymic action.

This conception of the form in which the thyroid hormone is stored may be over-simplified. The question of the relative biological potency of thyroglobulin from the thyroid gland, and free thyroxine, is still unsettled. The most recent contribution to this question comes from Frieden and Winzler¹¹. They compared the effect of various natural and artificial iodo-proteins in preventing goitrogenesis in rats treated with thiouracil. The biological potency of the natural thyroglobulin preparations was found to be decidedly higher than would be expected from their thyroxine content, as chemically determined. The implications of these findings, if they are to be accepted in spite of other contradictory observations (which the authors review), are far from clear. They are presented here only to remind the reader that the idea that thyroxine (the circulating form of the hormone) is stored in the thyroid by being incorporated into the molecule of a protein (thyroglobin), may not be a wholly adequate account of the situation. It is, however, a highly convenient working hypothesis, and is accepted here for purposes of further discussion.

The analogy of the artificial iodoproteins suggests that iodine in the thyroid combines directly with part of a protein, so that the resulting thyroxine molecule is from its inception built into the structure of the parent protein molecule. The part of the protein with which the iodine combines is presumably the amino-acid tyrosine. Harington¹² pictures the synthesis of thyroxine as occurring in two stages: first the iodination of tyrosine to form diiodotyrosine, then the condensation of two diiodotyrosine molecules to form thyroxine. Oxidation is required for both stages, the first step being the conversion of iodide to iodine. Since, in the presence of thiouracil, iodide can no longer be brought into organic combination in the thyroid, it follows that thiouracil prevents the first step in the process, the iodination of tyrosine. However, Dempsey and Astwood¹³ provided evidence that, even if this first step could occur, thiouracil would prevent the second, for diiodotyrosine was extremely ineffective in preventing thyroid enlargement in rats treated with thiouracil. This finding has been confirmed by Frieden and Winzler¹¹.

It may be assumed that thiouracil prevents the iodination of protein. It could do this by preventing the oxidation of iodide to iodine, or by itself combining with the free iodine after oxidation had occurred. The nature of the enzyme system which effects this oxidation is not known. Schachner, Franklin and Chaikoff¹⁴ showed that the conversion of radio-

active iodine by isolated thyroid slices to diiodotyrosine and thyroxine is prevented by cyanide, azide, sulphide and carbon monoxide. Since all these agents inhibit the cytochrome-oxidase system, they concluded that this system was essential for the production of thyroxine, and suggested that its function was the oxidation of iodide to iodine. However, this enzyme system is of such general importance to the cell that its inhibition might well prevent thyroxine formation indirectly, even if some other enzyme were responsible for the actual oxidation of iodide.

Another system which could oxidise iodide is the hydrogen peroxide—peroxidase system (Westerfield and Lowe¹⁵). Keston¹⁶ showed that hydrogen peroxide and peroxidase accelerate the iodination of casein *in vitro*. Although there is histochemical evidence for the presence of this enzyme in the rat thyroid¹⁷, Glock¹⁸ and Astwood⁴ were quite unable to demonstrate it by chemical means.

Whatever the nature of the enzyme system concerned there are at least three ways in which the anti-thyroid drugs might interfere with its action: they might poison the enzyme itself, they might compete with iodide as substrate or they might themselves combine directly with iodine and thus prevent its combination with protein. If cytochrome-cytochrome oxidase is the effective oxidative system, then the evidence that the anti-thyroid drugs act by enzyme-poisoning is not good. It is true that Paschkis and others¹⁹ claimed that thiouracil inhibits the cytochrome oxidase system of the thyroid, both when added to isolated thyroid slices and when given to the intact animal. However, this enzyme system is responsible for such a large proportion of normal tissue respiration that this finding conflicts with Lerner and Chaikoff's²⁰ observation that thiourea, thiouracil and sulphonamides have no effect on the oxygen consumption of isolated thyroid slices. Indeed, the thyroids of rats fed on thiouracil have been found to consume more oxygen than normal glands (Jandorff and Williams²¹), presumably as a result of their relatively greater cell mass. It is scarcely likely that this could have occurred if the cytochrome oxidase of the thyroid had been inhibited to any serious extent. McShan and others²² and Dempsey¹⁷ were unable to confirm the inhibition of cytochrome oxidase by thiouracil.

Dempsey¹⁷ has claimed that peroxidase activity in the follicular cells of the thyroid (as demonstrated by the benzidine reaction) is inhibited by thiouracil. Glock¹⁸ found that thiouracil and thiourea inhibited the actions of peroxidase and hydrogen peroxide on pyrogallol; but, as has been mentioned before, neither she nor Astwood⁴ could find any evidence that peroxidase exists in the thyroid gland. Randall²³ examined the behaviour of anti-thyroid compounds *in vitro* in the presence of hydrogen peroxide and peroxidase. He showed that the effect of these compounds on peroxidase cannot be studied by methods involving the oxidation of dyes (*para*-aminobenzoic acid red, 2:6-dichlorophenolindophenol and benzidine blue), because they are such strong reducing agents that they decolourise the dyestuffs. Dempsey's¹⁷ histochemical evidence of

peroxidase inhibition by thiouracil is therefore invalid. Furthermore, if the anti-thyroid compounds are added to hydrogen peroxide and peroxidase it is found that the hydrogen peroxide rapidly disappears. Under these conditions the anti-thyroid compounds do not inhibit the peroxide-peroxidase system; they act as substrate for it. Randall suggests, as a possible mode of action of these compounds *in vivo*, that they may act as reducing agents by competing for hydrogen peroxide as it is formed, and thus prevent it from taking part in the oxidation of iodide. It must, however, be remembered that there is no good evidence that the peroxide-peroxidase system is concerned in thyroxine formation *in vivo*.

Thus the hypothesis that thiouracil and its derivatives act by poisoning oxidative enzymes is unnecessary, and the evidence in its favour can no longer be regarded as substantial. The reducing powers of these substances are such that they could easily act as substrate in competition with iodide. Equally, they could act by combining directly with free iodine as it is formed. The difference between these last two theories is not, from the physiological point of view, very great, for both state that the ability of the thiol compounds to prevent the synthesis of thyroxine is attributable to their reducing properties. However, since it has not been demonstrated that any peroxidase exists in the thyroid, the theory of direct combination with iodine seems the more acceptable.

Direct evidence in favour of this last theory was first presented by Campbell, Landgrebe and Morgan²⁴, who showed that thiourea reacted with iodine to give formamidine disulphide. If this reaction occurs in the thyroid it would have the effect of keeping the iodine there in the reduced form. Later, Miller, Roblin and Astwood²⁵ showed that thiouracil was similarly oxidised by iodine to its disulphide. They examined a series of other compounds in a similar way and found that there was a general correlation (with a few exceptions) between the speed of the reaction and the number of molecular equivalents of iodine with which a compound would react, and its goitrogenic power. Some reducing substances, however, such as glutathione, had a marked power of reducing iodine, but no goitrogenic activity. The same authors showed that thiouracil could inhibit the iodination of casein and tyrosine *in vitro*.

More recently, Pitt-Rivers²⁶ has shown that thiouracil and similar compounds can inhibit the *in vitro* formation of acetylthyroxine from acetyldiiodotyrosine. She demonstrated that when one of these compounds (tetramethylthiourea) is incubated with acetyldiiodotyrosine, iodine is slowly liberated and oxidizes the thiourea compound. The iodine is thus prevented from oxidising the acetyldiiodotyrosine, and no acetylthyroxine is formed. The ability of various compounds to prevent the formation of acetylthyroxine ran roughly parallel to their ability to prevent the synthesis of thyroxine *in vivo*. Thus the iodine-combining power of thiouracil and related compounds provides a satisfactory explanation of their action in preventing the formation of thyroxine in the living animal, even when diiodotyrosine is provided.

THE RELATIVE EFFECTIVENESS OF THE ANTI-THYROID DRUGS

Two motives have prompted the various surveys of large numbers of chemical compounds for their ability to inhibit thyroxine synthesis: on the one hand, the desire to define the chemical grouping responsible for the physiological effect; and on the other hand, to find the most suitable substance for the control of thyrotoxicosis. The first of these objectives has not been attained; indeed, it is not likely that it will be attained, at least in terms of conventional organic chemistry. The search for useful therapeutic substances has met with more success.

The methods which have been evolved to compare the various substances belong to three broad types: the purely chemical, the use of slices of thyroid tissue *in vitro*, and tests on intact animals. Miller, Roblin and Astwood²⁵ showed that thiouracil could inhibit the iodination of casein *in vitro*, by itself reacting with the iodine present, and examined the ability of a number of anti-thyroid compounds to react with iodine. Pitt-Rivers²⁶ compared the ability of various anti-thyroid compounds to prevent the conversion of acetyldiiodotyrosine to acetylthyroxine. The results of these two methods are in reasonably good agreement with each other and with tests on animals. Thus the thiourea derivatives are a great deal more active in both tests than are the sulphonamide derivatives. Thiouracil is decidedly more active than thiourea. The principal discrepancy is in the case of 6-aminothiouracil. According to Pitt-Rivers, this substance is much less active than thiouracil, but Miller, Roblin and Astwood found it equally reactive with iodine. In the rat²⁷ and in man²⁸ it showed no detectable anti-thyroid activity.

The reducing action of a number of substances in the presence of a hydrogen peroxide-peroxidase system was examined by Randall²³. It was again evident that there was a rough correlation with the results of animal experiments. The sulphonamides were weak reducing agents, and thiouracil was more active than thiourea.

Chaikoff and his collaborators^{29,2} have studied the effect of various substances on the conversion of radiiodide into radio-diiodotyrosine and radio-thyroxine by isolated slices of thyroid. These studies were very valuable as providing the first evidence that the thiouracil group acted by preventing the synthesis of thyroxine and not the entry of iodide into the thyroid cell. For purposes of comparison of various anti-thyroid substances they are less useful, since for the most part substances were only tested at a single concentration. At the concentration generally used by these workers (10^{-3} M) both thiouracil and thiourea inhibited the formation of radio-thyroxine virtually completely; in the case of the sulphonamides and para-aminobenzoic acid the degree of inhibition was a little less.

For tests on intact animals, rats and chicks have been most commonly used. The results obtained in laboratory animals have been extended by studies of the effects of anti-thyroid compounds on radio-iodine up-

take by the thyroid in the normal human subject, and by clinical trials. Using the rat as test animal, Astwood and his colleagues alone have examined more than 300 substances^{8,27}. The method used has been to administer the substance under investigation either in the food or the drinking water for 10 days. The animals are then killed, and the thyroids weighed and either examined histologically or kept for estimation of the iodine content. These methods have fully justified themselves as a means of rapid examination of a large number of possible anti-thyroid substances. The results obtained, however, should not be accepted uncritically as giving a quantitative measure of the ability of a substance to prevent the synthesis of thyroxine. For thyroid weight, histological appearances and iodine content in this test depend not only on the completeness with which thyroxine synthesis is inhibited, but also on the rate at which preformed thyroxine disappears from the gland. This is in turn determined by such factors as temperature and perhaps food intake¹³. The presence of many of the anti-thyroid substances in the diet diminishes food intake and slows growth. Astwood's²⁷ figures show that even a substance as little toxic as propylthiouracil causes a marked fall in growth-rate at the higher dose levels. This fall in growth-rate is associated with a less marked increase in thyroid weight and a less marked fall in thyroid iodine. Determination of total thyroid iodine is also open to fallacy because it depends on dietary iodide, which may change unexpectedly. When these known sources of error can be excluded there still remains the unexplained phenomenon (discussed by Astwood⁴) of the variations in the shape of the dosage curve from one substance to another. The thiobarbiturates, for instance, give an increased thyroid weight and decreased thyroid iodine at a low dosage; with increasing dosage, however, these effects do not increase proportionately, so that it is never possible to produce a really large goitre with these drugs. This result does not seem to be due to decreased food intake²⁷.

For the reasons given above, this type of test in rats is not suitable for exact quantitative work. Observation of the effect of anti-thyroid compounds on the uptake of radio-iodine by the thyroid is in some ways more satisfactory. The results of such tests are at present available for the normal human subject. The method employed by Stanley and Astwood²⁸ was to give tracer doses of I^{131} (without carrier) and then measure the increase in radio-activity over the neck. By plotting the count against the square root of time an approximation to a straight line could be obtained, for a sufficiently long period. The drug to be tested was then administered and the amount of downward deflection of the straight line noted. The responses were graded by degree and duration into 5 classes of increasing inhibition. The method is open to the criticism that the final measurement is not a quantitative one. Nevertheless it has yielded interesting results, and is a much more useful test for clinical purposes in that the experimental animal used is man.

The results obtained in this way show the sulphonamides to be even weaker, relative to the thiourea group, than in other tests; in fact,

THE ANTI-THYROID COMPOUNDS

sulphadiazine had no detectable anti-thyroid action in a 500 mg. dose. This agrees well with the experience gained in the late war, when large numbers of American troops were given sulphonamides for long periods, as a prophylaxis against streptococcal infections; no goitre was reported. This finding is in contrast with tests in the rat²⁷, which show sulphonamides to be about as active as thiourea.

Thiourea, on the other hand, showed a much greater activity than would have been expected on the basis of its action *in vitro* and in the rat. In Stanley and Astwood's tests in man thiourea proved as potent as thiouracil. The smaller effect in the rat may perhaps be explained by the great loss of appetite which thiourea causes in this animal. Since thiourea is more soluble than thiouracil, it may be that it penetrates more easily into the thyroid, and thus has a greater potency in Stanley and Astwood's tests than would have been predicted from its behaviour *in vitro*.

The results obtained with 6-substituted thiouracils are also of interest. In the rat, thiouracil, 6-methylthiouracil and 6-*n*-propylthiouracil have relative potencies in the order 1:1:11. In man, the order is 1:2:0.75. The reason for the discrepancy is not apparent. Propylthiouracil causes an initial loss of appetite and failure to grow in the rat to about the same extent as the other two compounds. The results of Stanley and Astwood's tests on these three compounds are in fairly good agreement with clinical experience; although the minimal doses necessary to give a full response have never been accurately determined, they are of the order of 200 mg. daily for methylthiouracil, and 300 to 400 mg. daily for the other two compounds.

The most potent substance tested by Stanley and Astwood was 2-mercapto-imidazole. This substance had 1.5 times the potency of thiouracil in the rat, but 10 times the potency in man. There are a number of less striking discrepancies between the values obtained for man and the rat, for which the original paper²⁸ should be consulted. It is sufficient here to say that the existence of such discrepancies must discourage any attempt to correlate anti-thyroid activity with detailed chemical structure.

The significance of these findings to the clinician is not very apparent. The mere fact that minute doses of a compound, such as 25 mg. of mercapto-imidazole, can cause prolonged inhibition of thyroxine synthesis, does not of itself mean that this is the ideal drug for clinical purposes. It might well be, for instance, that such a drug would produce more toxic reactions than another which had to be used in 10 or 20 times the dose, in order to inhibit thyroxine synthesis. Clinical reports on the practical utility of this drug are not yet available.

In fact, the number of *effective* anti-thyroid drugs available to the clinician is embarrassingly large. If a test could be devised which would estimate the proportion of "toxic reactions" to be expected clinically, it would be of great practical value. Unfortunately, no such test exists

at present. All our knowledge of toxic effects comes from clinical experience and is almost useless for purposes of comparison of one drug with another. The only common toxic reactions are drug fever, and rashes; the only dangerous toxic reaction is agranulocytosis. All three of these effects are classed as "idiosyncrasies"; that is to say, they only occur in certain subjects who are said to be "sensitive" to the drug in question. The only way we have of estimating the toxic properties of these drugs is to give them to a large number of subjects, and note the proportion who get these reactions. The accumulated clinical experience up to the present date shows that thiobarbital and aminothiazole are too toxic for routine use, and that thiouracil is more toxic than methylthiouracil or propylthiouracil. These are crude statements of clinical experience, and it is very desirable that they should be amplified by further pharmacological research. In this connection, the observations of Lehr³⁰ on toxic reactions with sulphonamides are of considerable interest. With these drugs also the common toxic reactions are drug fever and rashes. Lehr presents evidence to show that, although these reactions fall into the category of sensitisation phenomena—i.e., they only occur in susceptible subjects—their incidence still shows a relation to dosage. Since there appears to be a critical dosage, below which no reactions occur, he suggests that combinations of two or three different sulphonamides should be used. Since each sulphonamide would be given in a dose less than the critical one, no toxic reactions should occur. The use of such combinations has, he claims, reduced the incidence of toxic reactions in practice. He suggests that the same line of reasoning might well be tried with the anti-thyroid compounds.

It is always assumed that similar toxic reactions do not occur in laboratory animals. The present writer is not aware, however, that there has been any large-scale attempt to discover whether or not a *small proportion* of animals have, say, a transient bout of fever during the administration of thiouracil. It may be that we are too apt to assume that rats cannot display as much individuality as human beings. However, the stimulus to such investigations has to a large extent passed, since in the doses used at present methylthiouracil and propylthiouracil seldom cause alarming reactions.

THE SIGNIFICANCE OF NATURALLY OCCURRING ANTI-THYROID SUBSTANCES

It is established beyond all reasonable doubt that an extreme deficiency of iodine in the diet can cause goitre, and that the mechanism by which such goitre is produced is similar to that of the thiouracil goitre; that is to say, in the absence of iodine no thyroxine can be formed, hence a compensatory overactivity of the pituitary occurs, with consequent thyroid enlargement. It is also established that, even where iodine lack is not extreme, there is a general inverse relationship between iodine content of the soil and water, and the incidence of goitre. But this inverse

relationship does not always hold in detail. Hence, if the occurrence of simple goitre is to be fully explained, some other cause must be sought for which is sufficient to produce goitre in areas where the dietary iodine is not low enough to be goitrogenic by itself.

Since Chesney, Clawson and Webster³¹ first described, in 1928, the production of goitre in rabbits by feeding cabbage, a number of different foodstuffs have been found to cause goitre in animals. Among such foodstuffs are soya-beans, rape-seeds, turnips and peanuts. Some of the evidence is, however, curiously conflicting. Thus Chesney, Clawson and Webster's results were confirmed by McCarrison³² in India and Spence, Walker and Scowen³³ in this country, but Hercus and Purves³⁴ in New Zealand and Zeckwer³⁵ in U.S.A. found the goitrogenic properties of cabbage to be weak and uncertain. Webster, Marine and Cipra³⁶ explained these anomalies by showing that there were marked seasonal and year-to-year variations in the potency of cabbage. 1928-9 was a vintage year, for cabbage maturing in that winter produced palpable goitres in 7 to 10 days. Similarly, Hercus and Purves³⁴ found a great variation in the goitrogenic activity of turnips when tested on rabbits. Turnips from an area where outbreaks of congenital goitre in lambs had occurred caused large goitres in rabbits in 1933, but not in 1934.

Recently Greer and Astwood³⁸ have tested some 60 different foodstuffs for their ability to check the uptake of radio-iodine by the thyroids of normal human subjects. The technique was the same as that previously used by Stanley and Astwood²⁸ in their survey of the effect of various anti-thyroid substances in man. The activity of several members of the *Brassica* group of vegetables was confirmed. Cabbage and turnip were both active, but swedes (rutabaga) even more so. However, several members of other vegetable groups were also active, such as peaches, pears, strawberries, spinach, lettuce, peas, walnuts and carrots. In nearly all these cases much larger quantities were consumed in the test than would ever be eaten in normal circumstances. However, peanuts, filberts and swedes showed some effect in relatively small quantities. Of the animal products tested there was some indication of anti-thyroid activity in milk, liver and oysters. As with previous investigations there was a wide variation in the response to the same foodstuffs in different trials. A significant depression of radio-iodine uptake followed a mixed meal, consisting of raw carrots, swedes, lettuce, pears and milk.

The importance of these results is two-fold. In the first place they show that many foodstuffs can have an anti-thyroid effect in man as well as in laboratory animals. The response to anti-thyroid substances varies greatly from one species to another, so that this evidence is indispensable if a case is to be made out for foodstuffs as a cause of human goitre. Secondly, Greer and Astwood have shown that the range of foodstuffs in which anti-thyroid substances may be found is much greater than had previously been suspected.

Greer and Astwood have no further data to present on the nature of the naturally occurring anti-thyroid substance or substances. Such substances could belong either to the thiocyanate group, which prevents the uptake of iodide by the thyroid, or the thiouracil group, which prevent the synthesis of thyroxine. In previous animal experiments it had been found that cabbage leaves and soya-beans resembled the thiocyanates in that their effects were easily preventable by iodide, whereas those of rape-seeds, like thiouracil, were not. The mustard oils are derived from several members of the *Brassica* group, which contain a glycoside (sinigrin) and an enzyme, myrosin; in the presence of water these interact to form allyl isothiocyanate. However, isothiocyanates do not cause goitre in the rat²⁷.

In the presence of ammonia allyl isothiocyanate is readily converted to allylthiourea. It was for this reason that Kennedy³⁷ originally suspected that allylthiourea was the active principle in rape-seed. He demonstrated that allylthiourea could cause goitre in the rat. However, neither this nor any other active goitrogenic substance has actually been isolated from any foodstuff. The nature of the substance or substances which cause the effects noted by Greer and Astwood therefore remains unknown; in the absence of direct evidence the balance of probabilities seems to favour a member of the thiourea group, but the thiocyanates may also make a contribution.

It is difficult to assess the practical importance of these findings. In the case of many of the foodstuffs noted as giving a positive result the deflection of the iodine uptake curve was a very minor one, and was, moreover, often inconstant in different trials. It does not necessarily follow that such minor deflections are of any significance to the human organism. In the normal thyroid, with its ample reserves of stored hormone, partial inhibition of thyroxine synthesis would not be reflected by any change in blood thyroxine levels unless such inhibition had persisted continuously for long periods. Clinicians who have tried to produce myxœdema in subjects with normal thyroids for therapeutic reasons are well aware that many months of intensive treatment with anti-thyroid drugs are necessary before goitre results.

If foodstuffs are an important cause of goitre (apart from their iodine content) they are likely to be so when the iodine intake is already low, and when a highly abnormal diet is being consumed. Greer and Astwood³⁸ have noted the occurrence of goitre in individuals on vegetarian diets, or who developed a craving for certain foods, such as lettuce. Bastenie³⁹ has brought forward evidence which suggests that there was an increase in simple goitre in occupied Belgium during the last war. The population at that time lived on a largely vegetarian diet, in which *Brassica* roots were prominent. It is conceivable, as Bastenie suggested, that this diet was responsible both for the increase in simple goitre, and also for the apparent decrease in the severity of thyrotoxicosis, which he considered was occurring at the same time.

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RESEARCH PAPERS

ASSAY OF THE CURATIVE ACTION OF NEOARSPHENAMINE BY TIME-MORTALITY DATA

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THE commonest current methods employed for testing the action of neoarsphenamine are those in which the drug is injected into mice or rats already lightly or heavily infected with *Trypanosoma equiperdum* on a previous day. Blood specimens removed from every animal are examined daily, involving the counting of many squares of the counting-chamber, before deciding whether or not the animals are cured. These methods are laborious and time-consuming, and for this reason, and because of other disadvantages, Bülbring and Burn¹ proposed that the activity of a preparation should be estimated from the survival times of mice infected and treated on the same day. In the present work, this method has been extended and examined statistically.

METHOD

Blood taken from rats which had been infected 2 days earlier was diluted with 1 per cent. sodium citrate solution till it contained 7,000 trypanosomes in 1 microlitre. Mice weighing about 16 to 18 g. were infected by intraperitoneal injection with 0.5 ml. of this trypanosome suspension. Neoarsphenamine was injected intravenously in 0.2 per cent. solution within 2 hours from the time of infection. The doses were calculated in proportion to the body weight. The usual precautions to prevent the oxidation of the neoarsphenamine were taken.

RESULTS

The results of a preliminary pilot-experiment provided a curve relating dosage with survival time. Seven groups each of 15 infected mice were injected with graded doses of neoarsphenamine ranging from 13.9 $\mu\text{g./g.}$ of body weight upwards by steps of 20 per cent. From 2 days after the infection the mortality was noted every hour for 36 hours. After that, observations at night were discontinued. For the two

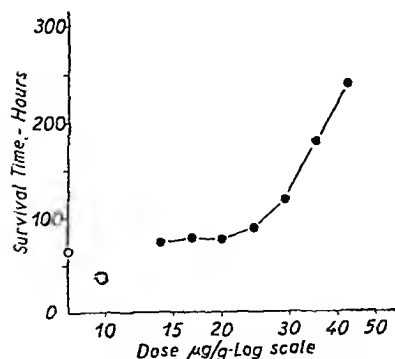


FIG. 1. Survival in hours from the time of infection for the median in every group.

Untreated. ● Treated.

highest doses the mortality was noted only once a day. A control group of 40 mice was followed at the same time. The survival time-

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dosage curve was drawn from the medians of the survival times in every group (see Fig. 1). All animals, except those receiving the highest dose, died, the last mouse surviving until the 15th day. In the highest dose group, i.e., those receiving 41.5 $\mu\text{g./g.}$, 3 animals survived, the last one dying on the 18th day. Since we rarely found any animals dying after this time it was decided that any mouse living beyond the 18th day should be counted as definitely cured.

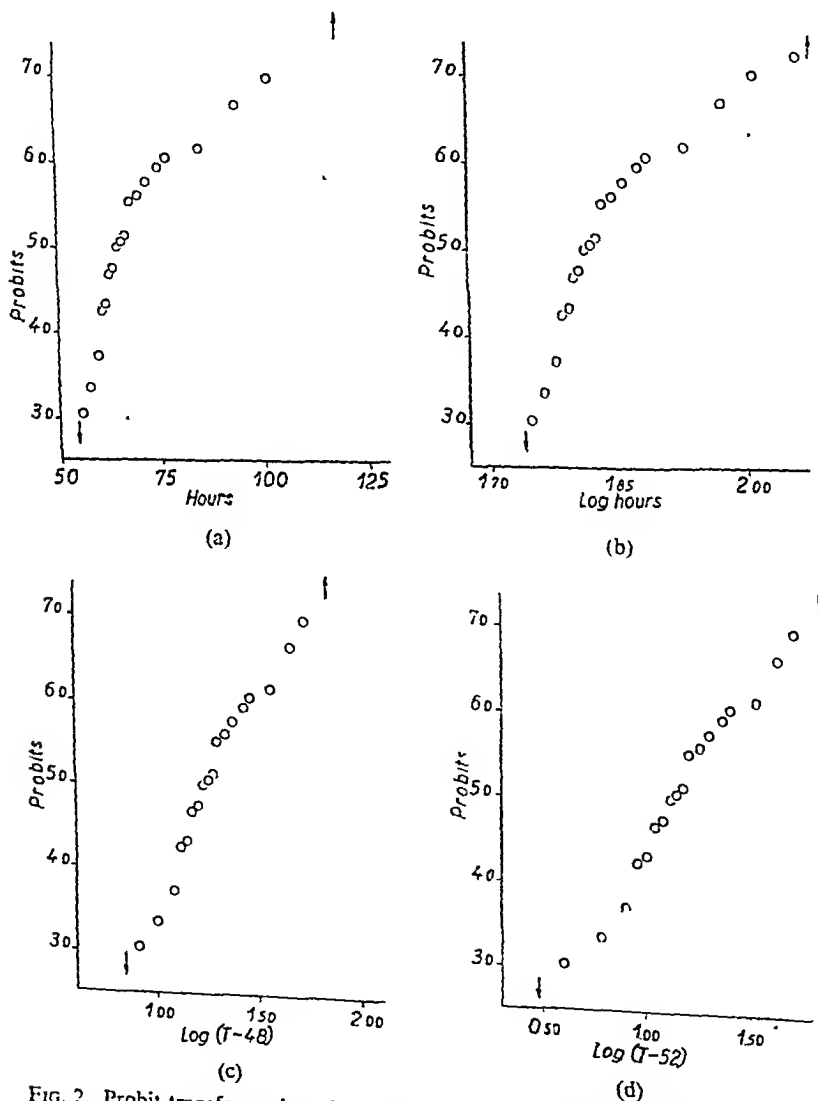


FIG. 2. Probit transformations for the survival times from the time of infection of the 40 infected control mice. (a) Survival time in hours; (b) Log survival time in hours; (c) Log (survival time in hours - 48); (d) Log (survival time in hours - 52). The times of the last observation with all living animals and those of the first observation with all dead animals are marked with arrows.

Using this basis we observed which function of survival time had a normal distribution. In toxicity tests in which the lethal time is delayed, some function of the time elapsing between the time of injecting the animal and its death can be found to be normally distributed, and the standard deviation of this function becomes a suitable measure of the varying sensitivity of the animals to the drug. Since the untreated infected controls show differences in their survival times, these were also determined and their limits of variation ascertained.

Of the 40 controls mentioned above, 4 animals died during periods when observations were more infrequent. For the other 36, the time of death could be stated to within an hour. The percentage of animals that had died up to the various times was transformed into probits according to Bliss². These values were then plotted on graph paper with the probits along one axis and the time or the logarithm of the time along the other, the time being measured from the moment when the animals were infected (see Fig. 2 a and b). If the plotted function of time had been normally distributed, the points should have fallen mainly along a straight line. In neither case did this happen, however, but the points fell along curved lines. That this was not accidental could be seen from similarly curved lines with corresponding probit transformations for the lower neoarsphenamine doses, in spite of the small number of animals in each one of these groups.

Every mouse was inoculated with about 3.5 million trypanosomes. In this way it received such a large number of trypanosomes that one hardly needs to take into account any differences in virulence between the infecting material of the different mice due to random variation. The dispersion of survival times will thus be mainly due to the host animals, i.e., the possibilities of growth for the trypanosomes in the different mice and the varying resistance of these to the fully developed infection. With intraperitoneal infection the conditions of growth may be regarded as nearly optimal, and so no great differences should exist between the different mice on this ground. This line of reasoning is confirmed by the results from the trypanosome counting method. In this the animals are used when the infection is very strong in the blood, that is, 2 days after being infected. Relatively few animals, however, need to be rejected on account of badly developed infection. In other words, full development of the infection is reached at approximately the same time by the majority of the infected animals. Consequently the differences in survival times would chiefly be due to the varying resistance of the mice against the fully developed infection. If such is the case some function of the time between the point when full infection is reached and the time of death might have a normal distribution.

The first control mouse died 55 hours after being infected. Evidently full infection must have been reached some time earlier, after which the remaining time of survival was influenced only by the resistance of the mouse. Times of 48 and 52 hours after infection were, therefore, chosen, as it was considered that full infection might have been attained

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in the control group at one of these periods. New probit diagrams were made with the logarithms of the times from these new starting points along one axis, that is, the logarithm of the survival time in hours minus 48 and hours minus 52 respectively. In both cases good agreement with a normal distribution was obtained (see Fig. 2 c and d). In view of the small amount of material the points on both figures appear to lie reasonably near a straight line.

Also for the mice treated with neoarsphenamine the logarithms of (survival time in hours-48) seemed to be normally distributed for every dose. As the groups were rather small, on a later occasion 2 groups with 40 infected mice in each were treated with doses of 20 and 31 g./g. of body weight respectively. In these groups, too, no certain deviation from the formula just mentioned could be found, the points lying fairly well along a straight line in the probit diagram. On this occasion a certain change in sensitivity to the neoarsphenamine was observed as compared to that shown in Figure 1 since half of the animals in these two groups died after 108 and 218 hours.

If this transformation of the primary values, i.e., the logarithm of (survival time in hours-48) proves to be generally useful for experiments of this kind in different laboratories, the mice of a group in different tests or at different times should as a rule be approximately normally distributed when their times of survival are transformed in this manner. The values given by Bülbring and Burn and also those found in earlier experiments from this laboratory, lend support to this contention. These figures confirm, among other things, that the maximum mortality of the controls as well as that of the animals treated with small neoarsphenamine doses occurs during the night between the second and third day after the infection.

TABLE I

Reading Time		Time in Hours After the Infection (T)	Class Limit Log (T-48)	Class Width	Class Middle	
Day after Infection	Time of Day					
2	22 o'clock	...	61	1.114	0.248	1.238
3	8 "	...	71	1.362	0.104	1.414
3	14 ¹⁵ "	...	77.25	1.466	0.102	1.517
3	22 "	...	85	1.568	0.104	1.620
4	8 "	...	95	1.672	0.104	1.724
4	20 ¹⁵ "	...	107.75	1.776	0.105	1.828
5	13 "	...	124	1.881	0.104	1.933
6	9 ¹⁵ "	...	144.5	1.985	0.098	2.034
7	10 "	...	169	2.083	0.099	2.132
8	17 "	...	200	2.182	0.104	2.234
10	10 "	...	241	2.286	0.103	2.337
12	14 "	...	293	2.389	0.105	2.442
15	9 "	...	360	2.494	0.099	2.544
18	17 "	...	440	2.593	0.103	*2.645

* Calculated with the class width taken as 0.103, which is the mean of all class widths except the first

As it thus seems that the logarithm of (survival time in hours-48) in practice may be taken as normally distributed in the different dosage groups, due regard should be given to this fact in the spacing of the reading times. The following example shows how this may be performed so that the intervals between the observations are as far as possible equally large when expressed in the normally distributed function, i.e., log. (time in hours between the time of infection and the observation -48). Even allowing for this, the readings can be so arranged that most of them are made during the normal working day.

EXAMPLE

Groups of 20 newly infected mice, which had been kept in the laboratory for over a week before this experiment were injected intravenously with doses of 24 and 30 $\mu\text{g./g.}$ of body weight of the International Standard and of a commercial preparation respectively, both in 0.3 per cent. solution. The mice were infected at 9 o'clock in the morning and the injections of neoarsphenamine were made within the following 2 hours. The readings were performed according to Table I. The times when the mice were found dead are given in Table IIa and the corresponding class middle for each animal in Table IIb. Two methods of analysis of the data are now available.

TABLE IIa
RESULTS. NUMBER OF DEAD MICE

Reading Time					Standard		Test Preparation	
Day	Time of day				24 $\mu\text{g./g.}$ (S_L)	30 $\mu\text{g./g.}$ (S_H)	24 $\mu\text{g./g.}$ (U_L)	30 $\mu\text{g./g.}$ (U_H)
2	22 o'clock				
3	8	"			4	
3	14 ¹⁵	"			7	2
3	22	"	1		2	4
4	8	"	4		5	5
4	20 ¹⁵	"	8	3	2	6
5	13	"	2	6		2
6	9 ¹⁰	"	4	1		
7	10	"		2		
8	17	"	1	5		1
10	10	"		2		
12	14	"				
15	9	"				
18	17	"				
Surviving after the 18th day						1		

The simplest method is to calculate the means, standard deviations, and standard errors of the means in the usual manner for each group as in Table IIB t-analysis for the differences between the means of S_H and S_L and between U_H and U_L shows in the first case $P < 0.001$ and in the second case $P = 0.001$. The differences are therefore not attributable to random variation alone. This shows that the test really is sensitive to a difference in dosage of 20 per cent. For the difference between the means of S_L and U_H $P = 0.05$, i.e. the larger test dose has smaller effect than the smaller standard dose.

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TABLE IIb

RESULTS TRANSFORMED

CLASS MEANS FROM TABLE I FOR THE DEATH OF EACH MOUSE

Mouse			Standard		Test Preparation	
Number	24 $\mu\text{g./g. (St.)}$	30 $\mu\text{g./g. (Sn)}$	24 $\mu\text{g./g. (St.)}$	30 $\mu\text{g./g. (Un)}$
1	1.517	1.724	1.238	1.414
2	1.620	1.724	1.238	1.414
3	1.620	1.724	1.238	1.517
4	1.620	1.828	1.238	1.517
5	1.620	1.828	1.414	1.517
6	1.724	1.828	1.414	1.517
7	1.724	1.828	1.414	1.620
8	1.724	1.828	1.414	1.620
9	1.724	1.933	1.414	1.620
10	1.724	2.034	1.414	1.620
11	1.724	2.034	1.517	1.724
12	1.724	2.132	1.517	1.724
13	1.828	2.132	1.620	1.724
14	1.828	2.132	1.620	1.724
15	1.828	2.132	1.620	1.724
16	1.828	2.132	1.620	1.724
17	1.933	2.234	1.620	1.828
18	1.933	2.234	1.724	1.828
19	2.132	2.645	1.724	2.132
20	2.132	2.645	1.724	2.132
Total			35.099	39.914	29.432	33.128
Mean			1.75495	1.9957	1.4716	1.6564
Standard deviation			0.138	0.232	0.159	0.164
Standard error of the mean			0.031	0.052	0.036	0.037
Total S			...	75.013	Total U	62.560

More information, however, may be extracted from the material if it is subjected to variance analysis (Fisher³), the results of which are shown in Table III.

TABLE III
ANALYSIS OF VARIANCE. DATA OF TABLE IIb

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between samples	1	1.9385	1.93850	61.74	<0.001
Between doses of same substance	2	0.9211	0.46055	14.67	<0.001
Random sampling	76	2.3867	0.03140	—	—
Total	79	5.2463	—	—	—

Slope, 2.18

Potency, 0.72

Fiducial limits, 0.580 to 0.809
Percentage limits, 80.5—112.0

(P = 0.05).

The slopes of the dosage-response lines were calculated and found to be homogeneous. It was therefore legitimate to calculate a combined slope and to use this value in order to obtain the potency of the unknown in terms of the standard from the equation provided by Gaddum⁴, and the fiducial limits of error of the estimated potency from the equation provided by Fieller⁵.

Summarising the results, the unknown preparation had a potency of 0.72 of the standard with fiducial limits of error of 0.580 to 0.809 for P = 0.05.

When material is grouped it is desirable to group it within narrow class limits, i.e. to group it in many classes, in order to extract as much

information as possible. In this case the number of classes directly depends on the frequency of the readings; many times of observations allow many classes with narrow class limits, whereas few readings allow only a few classes and widely spaced class limits. The number of the observations, however, is limited by the fact that in practice it is hardly feasible to make observations during the night. An increase of the number of readings during the day but none during the night implies alternating small and large intervals between the observations, with varying class widths, and this naturally gives no increased precision.

This could also be verified in the material from our example in which more readings were made during the days than are recorded in the tables. The number of readings in the example is near the maximum possible in practice with approximately equally spaced intervals. The fact that the first class has a much larger width than the rest has no great importance as in any case the doses of neoarsphenamine must be so large that only single animals die here.

One objection that has been raised against using survival times for estimating the curative action is that one does not know if the death of the animal is due to the infection. A certain control of this, however, exists. No deaths are caused by the infection on the day when the animals are infected or on the first or even on the greater part of the second day after infection. If the animals die during this time, it is due either to technical faults, which should be few with proper technique, or to non-specific deaths. Mice dying during this time must therefore be excluded from the analysis. When the groups are not too small and the times of survival not too long, the death of one or two mice during this period does not appreciably alter the accuracy of the test. If more mice die, it shows that they were in bad condition and the assay must be rejected. It has been found convenient to keep the mice in the laboratory for some days before they are used in order to make sure that they are in good condition.

When it is desired accurately to determine the curative potency of a preparation, the calculations will be performed as indicated in the example quoted above. Often, however, it is only necessary to ascertain that the potency of the test preparation is not less than that of the standard or of a certain proportion of it. The doses to be used should give a clearly prolonged survival time without making the test unwieldy. In our experiments a dose of 24 to 30 $\mu\text{g/g.}$ of body weight fulfilled these conditions. As a control of the sensitivity of the method a weaker dose of the standard was given as well, by way of example say 20 per cent. weaker. In a successful experiment a difference between the two should be evident.

PRACTICAL PERFORMANCE OF A TEST

Guided by these principles and by the aforementioned results, the test is performed as follows: 60 mice which have been kept in the laboratory for a week are infected intraperitoneally with 0.5 ml. per mouse of a trypanosome suspension containing 7,000 trypanosomes per

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microlitre. This is obtained from the blood of rats that have been infected 2 days earlier. The trypanosomes are counted in a counting chamber and the blood is diluted with 1 per cent. sodium citrate solution to the desired concentration. The infection is performed at 9 o'clock in the morning. Within the next hour the mice are injected intravenously with the different doses of the 0.3 per cent. neoarsphenamine solutions. During the preparation of the solutions the usual precautions against oxidation are observed (*see* Burn⁶). 20 mice receive 24 μ g. and 20 mice 30 μ g./g. of body weight of the standard and 20 mice receive the dose of the test preparation that is to be compared with the higher standard dose, and which must not be less potent than this standard dose if the preparation is to pass the test. The mice are observed at 16 o'clock on the second day after the infection. Those which have died are rejected and excluded from the calculations. After that the readings of the mortality are spaced according to the times in Table 1. The mean and the standard error of the mean are calculated for every group, the value for every animal being that of the corresponding class middle seen in the table. Thus, if an animal has died only on the fourth day at 8 o'clock its value is 1.620. The preparation passes the test if t-analysis shows that it is stronger or not weaker than the larger standard dose. In the latter case, however, a significant difference between the standard doses must exist, otherwise the test must be repeated. If t-analysis shows that the test preparation is significantly weaker than the larger standard dose it is rejected.

DISCUSSION

When testing substances on animals, it can be shown in many cases that the logarithm of the duration of the effect or the logarithm of the time till the effect appears is approximately normally distributed (Bliss², Goodwin and Marshall⁷, Goldberg⁸ and others). A close study of this question, however, may reveal that a more complicated function of the time has a normal distribution (Ipsen⁹). On the other hand, it is sufficient to have an approximate knowledge of the kind of function that is normally distributed when grouping the observations as the laws for calculating the means, standard errors and t-values are also applicable to a number of different distributions, more or less deviating from the normal. The grouping of the observations advocated in this paper seems to be more rational than making one or two readings every day for a limited number of days as proposed by Bülbring and Burn¹ and Goodwin¹⁰ or for a longer times according to the method of Chen, Geiling and MacHatton¹¹. A further advantage is that an estimate of the error may be obtained for every dose, so extracting all information inherent in the material. A comparison with the results of Hawking¹² shows that, whereas in the trypanosome counting method every animal gives only a qualitative expression for the strength of the preparation in this method, the survival times are quantitative estimates of the strength of the drug, and thus furnish more detailed information.

information as possible. In this case the number of classes directly depends on the frequency of the readings; many times of observations allow many classes with narrow class limits, whereas few readings allow only a few classes and widely spaced class limits. The number of the observations, however, is limited by the fact that in practice it is hardly feasible to make observations during the night. An increase of the number of readings during the day but none during the night implies alternating small and large intervals between the observations, with varying class widths, and this naturally gives no increased precision.

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THE DETERMINATION OF ALKALOIDS BY EXCHANGE OF IONS

BY A. JINDRA

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THE development of research on substances serving for the exchange of ions has been going on for about a century. Research workers were at first dependent only on substances of natural origin, but in the years 1910 to 1930 many experiments were made towards preparing suitable synthetic products. In 1935, Adams and Holmes¹ prepared a new type of artificial resin, and thus, with this new group of substances, started development along new lines. Griessbach² examined in detail the composition, action and theory of ion-exchange substances, also their preparation and the determination of their activity, and explained their wide use for metallurgical and chemical purposes, especially as water softeners and salt-removers. In principle we are concerned with two groups of artificial resins. The first includes the condensation products of formaldehyde with aromatic acids, chiefly polyhydric phenols, and natural products of the tannin type, these condensation products serving as exchangers of cations, or eventually of hydrogen ions. The second group is formed by the condensation products of aromatic amines with aldehydes, these products serving for the exchange of anions, including hydroxyl ions. Hesse³ deals, under the heading of chromatographic determination, with the exchange of ions in some detail, from both theoretical and practical standpoints. Similarly, Myers⁴ sees in the exchange of ions only one of the methods of chromatographic determination (see also Zechmeister⁵).

Samuelson⁶ has contributed much to our knowledge of the use of these resins for the exchange of ions, especially in inorganic analysis. The *Encyclopædia of Chemical Technology* 1947 gives information of the use of synthetic resins in obtaining alkaloids of natural origin, such as quinine and nicotine, and details of the literature are given.

Ungerer⁷ has studied the adsorption of salts of organic bases, and to some extent alkaloids, on calcium permutite, and has ascertained that certain alkaloids, which are not easily soluble in water, are strongly adsorbed from aqueous solutions of their salts, due to the action of the slightly alkaline calcium permutite in precipitating the free base. In an alcoholic medium the adsorption proceeds in the normal way.

The idea of using the chromatographic adsorption method for the quantitative determination of alkaloids was realised soon after the revival of Tswett's method by Valentin, Franck and Merz^{8,9,10,11}, who describe the technique of adsorption on a column of aluminium oxide, and the working methods for determining the strength of various galenicals containing alkaloids. They have continued their work for several years, and give preference to the chromatographic method over

SUMMARY

The basis for using time-mortality data in estimating the curative effect of neoarsphenamine is examined. It is shown how the survival times may be transformed so that they become approximately normally distributed. A routine test has been designed on these lines.

The author is indebted to Dr. W. L. M. Perry, of the National Institute for Medical Research, London, for suggesting the treatment of the material with variance analysis and also for its application to the data.

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This prepared material was poured, together with a little water, into the adsorption tube, whose lower end was closed with a small wad of cotton wool, so as to ensure that the adsorbent material settled firmly in place, and did not tend to form layers when subjected to a steady flow of water. When a sufficient amount of material was in the tube, it was further washed with water, and secured by a second plug of cotton wool. The adsorbent was thus evenly distributed in a column about 5 cm. high, water flowing through it at a speed of about 100 to 120 drops per minute, under its own pressure.

Such a column has to be regenerated before and after each analysis. With Amberlite IR-4B this is carried out by means of a 4 per cent. solution of sodium carbonate. In determining alkaloids about 50 ml. of such a solution was sufficient for each regeneration. The excess of sodium carbonate was removed by washing with boiled distilled water until the washings gave no reaction with phenolphthalein. Usually about 300 to 400 ml. of water was sufficient. It was necessary always to maintain a layer of a few ml. of water above the adsorbent column in order to avoid the drying and packing of the material. When ceasing work the upper end of the tube was closed with a rubber stopper. If working with adsorption in a medium other than water, e.g. in alcohol, the adsorption tube and column should be washed out before the experiment with a few ml. of the solvent used.

The principal condition for success in the analysis of alkaloidal salts is the use of a solvent in which both the alkaloidal salt and the free base are readily soluble. Numerous experiments showed that it was not possible to obtain satisfactory results under any other conditions. Thus, for example, when working with strychnine nitrate in aqueous solution, the free base is precipitated on the adsorbent, and although strychnine is sufficiently soluble in ethyl alcohol, it was never possible, even using hot ethyl alcohol, to wash it out of the column quantitatively; and the results were always some 10 per cent. too low, even when using a considerable excess of solvent. This case is interesting since workers using aluminium oxide as adsorbent obtained quantitative extraction of the base from the adsorbent using comparatively small quantities of alcohol. For the examples used in the course of the present work, ethyl alcohol was found to be suitable, and in the case of morphine, methyl alcohol. In most cases the speed of flow of the liquid through the column is not important, and good results may be obtained with a speed of flow of 90 to 120 drops per minute. Only in the analysis of atropine sulphate did we obtain results rather lower than according to theory, and by reducing the speed of flow to 30 to 40 drops per minute we obtained satisfactory results. The speed of flow may be readily controlled by means of a rubber stopper in the adsorption tube in the neck of the Erlenmeyer flask in which the liquid is collected. After the adsorption is completed the tube and vessel are washed out with about 50 ml. of warm solvent (ethyl or methyl alcohol). This quantity of solvent may be regarded as sufficient for the alkaloids analysed, and

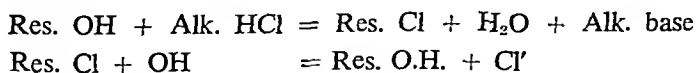
those used as standard in the pharmacopœias, the advantages lying chiefly in the smaller quantities of material required for analysis, the simpler technique, and the shorter time required for the analysis.

The most recent workers to direct their attention to chromatographic analysis of alkaloidal salts and to the suitability of aluminium oxide for such analyses are Reimers, Gottlieb and Christensen¹². They passed an ethyl alcohol solution of alkaloidal salts through a column of 10 g. of aluminium oxide, in a tube of 10 mm. diameter and 22 cm. height. The alkaloids were then washed out of the adsorbent with 25 ml. of ethyl alcohol (86 per cent.), and determined by direct titration in ethyl alcohol, using a suitable indicator in accordance with the experience of Kolthoff¹³ and Baggesgaard-Rasmussen and Reimers¹⁴.

The object of the work now to be described was first to ascertain whether artificial resins can be used as adsorbents for the determination of alkaloids, and then, if successful results were obtained, to investigate whether they could be applied to the determination of the alkaloidal content of galenical preparations. In the experimental part these questions are answered, the first very definitely in the affirmative, and the second with certain reservations. Nevertheless, even here certain definite results have been obtained, and the way indicated for further work.

EXPERIMENTAL

As an ion-exchanging adsorbent material, Amberlite IR-4B, manufactured by the Resinous Products and Chemical Company of Philadelphia, U.S.A., was selected, this being suitable for anion exchange. Attempts to find a basic exchanger suitable for quantitative work proved a failure, though such adsorbents gave excellent results when used for the isolation of alkaloids. The reactions in the adsorbing column were as follows:



The technique of the method has already been described in detail. Here only a brief description of the preparation of the adsorption apparatus is necessary since the whole was of the simplest type.

A tube of ordinary glass of 0.5 cm. diameter was used, which, at some 20 cm. from its end, was drawn out into a short narrow outlet tube, which was fitted into a rubber stopper, and the whole tube was thus attached to a suction flask.

It was necessary to crush the adsorption material, and it was found best to crush it and then leave it standing under water in a beaker for a day or two. During this period it was several times stirred up, and, when it had settled again, the water was changed. The light yellow powder was thus removed, and a material obtained of constant grain size, which plays a certain role in obtaining a perfect exchange of ions.

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as indicator a mixture of 10 drops of solution of methyl red with 2 drops of solution of methylene blue. To ascertain the equivalence point the mixture was diluted to 100 ml. with distilled water. The colour change from green through blue to violet is sharp.

For quinine and cinchonine the alkaloidal base was used for analyses, being dissolved by the addition of an equivalent quantity of hydrochloric acid and 10 ml. of water, the solution being made up to 100 ml. with ethyl alcohol. For the determination of morphine and quinine methyl alcohol solutions were used. Strychnine nitrate was dissolved in ethyl alcohol (75 per cent.). The percentage content of alkaloids in the salts was ascertained experimentally by the Danish Pharmacopœia methods. This was necessary because in many cases impure compounds were used whose content differed considerably from theory. The results are shown in Table I.

TABLE I
DETERMINATIONS OF ALKALOIDAL SALTS

Alkaloidal Salt	Weighed Out	N/10 Hydrochloric Acid (f=1.029)	Found by Adsorption Method	Found by Danish Pharmacopœia Method
		ml	per cent	per cent
Strychnine nitrate	g			
	0.0950	2.17	83.36	84.14
	0.0950	2.16	82.92	
	0.1420	3.27	84.04	
Atropine sulphate	0.1691	2.23	41.74	40.40
	0.2184	2.73	39.48	
	0.2267	3.00	41.79	
Morphine hydrochloride	0.1395	3.37	75.24	75.91
	0.1395	3.38	75.46	
	0.1395	3.35	74.79	
Brucine hydrochloride	0.1261	2.47	84.37	84.29
	0.1261	2.48	84.71	
	0.0966	1.89	84.23	
Ephedrine sulphate	0.1556	5.05	58.52	58.86
	0.1556	4.98	57.70	
Quinine	0.1344	3.74	98.52	
	0.1344	3.78	99.57	
Cinchonine	0.0976	3.14	98.46	
	0.0976	3.13	98.15	

(b) DETERMINATION OF ALKALOIDAL CONTENT OF GALENICALS

The determination was carried out according to the same principles as for the alkaloidal salts. Tincture of ipecacuanha and tincture of nuxvomica were adsorbed direct, while fluid extract of cinchona and tincture of opium had the weighed quantity diluted with 25 ml. of ethyl alcohol (96 per cent.). The further procedure was the same as that detailed above. The colouring matter is adsorbed in the column only in small measure, and the solutions of the alkaloidal bases obtained are coloured. The equivalence point must be determined electrometrically with the aid of the glass and calomel electrodes. With tinctures of nuxvomica and ipecacuanha the potential change is, however, masked by the accom-

it was not thought necessary to ascertain the minimum quantity of solvent needed, since we were using a quantity appropriate to the amount of substance being determined.

Adsorption from galenicals was carried out in exactly the same manner, since the first experiments showed that it was unnecessary to add inorganic acids in advance, in order to prepare salts of the alkaloids with strong acids. The question of a suitable solvent again plays the same important role. The alcoholic solution of the galenicals lost a small amount of its colour in passing through the adsorbing column together with some of the accompanying substances. In regenerating the adsorbent material after each analysis we remove the colouring matter and accompanying matter retained by the adsorbent, so that it is not necessary to change the contents of the column after each analysis.

The actual determination of the alkaloidal bases was carried out by direct titration with hydrochloric acid. In determining alkaloidal salts, i.e., in colourless solutions, the indicator used was a mixture of methyl red and methylene blue, giving at the appropriate dilution with water (about 100 ml.) a sharp change from green to violet. When working with galenicals it was necessary to ascertain the equivalence point electrometrically, using very sensitive measuring instruments. The potential drop is masked in these cases by the accompanying substances (the more intensely coloured the solution, the more difficult is the determination of the equivalence point). It is interesting to compare the titration curve of strychnine in ethyl alcohol with that of the same quantity of strychnine in tincture of nux vomica, in which the masking effect under the conditions prevailing in the tincture is clearly evident. There are possibilities here for working with minimum quantities, and also of using micro-methods for the determination of alkaloids in various galenicals, which are being further investigated by the present author. The cause of the double result obtained with tincture of nux vomica as compared with the theoretical result (the equivalent used was the mean of the molecular weights of the two alkaloids) is also being investigated.

The time required to carry out the analysis is about the following:—filling of apparatus, 15 minutes; regeneration of adsorbent, 30 to 45 minutes; adsorption and washing out, 15 minutes. The total time required is from 1 hour to $1\frac{1}{4}$ hours, in addition to the time required for the titration.

(a) DETERMINATION OF ALKALOIDAL SALTS

An accurately weighed quantity of the alkaloidal salt (0.1 to 0.2 g.) was dissolved in 20 ml. of ethyl alcohol (96 per cent.), and adsorbed on the prepared column of Amberlite IR-4B synthetic resin. The vessel was then washed out several times with a total of 50 ml. of warm (50°C.) ethyl alcohol (96 per cent.), and the column washed out with the same solvent. The dissolved base in the alcoholic solution was then determined by titration with N/10 hydrochloric acid ($F = 1.029$), using

THE DETERMINATION OF ALKALOIDS

as indicator a mixture of 10 drops of solution of methyl red with 2 drops of solution of methylene blue. To ascertain the equivalence point the mixture was diluted to 100 ml. with distilled water. The colour change from green through blue to violet is sharp.

For quinine and cinchonine the alkaloidal base was used for analyses, being dissolved by the addition of an equivalent quantity of hydrochloric acid and 10 ml. of water, the solution being made up to 100 ml. with ethyl alcohol. For the determination of morphine and quinine methyl alcohol solutions were used. Strychnine nitrate was dissolved in ethyl alcohol (75 per cent.). The percentage content of alkaloids in the salts was ascertained experimentally by the Danish Pharmacopœia methods. This was necessary because in many cases impure compounds were used whose content differed considerably from theory. The results are shown in Table I.

TABLE I
DETERMINATIONS OF ALKALOIDAL SALTS

Alkaloidal Salt	Weighed Out	N/10 Hydrochloric Acid (f=1.029)	Found by Adsorption Method	Found by Danish Pharmacopœia Method
	g.	ml.	per cent.	per cent.
Strychnine nitrate ...	0.0950	2.17	83.36	84.14
	0.0950	2.16	82.92	
	0.1420	3.27	84.04	
Atropine sulphate ...	0.1691	2.23	41.74	40.40
	0.2184	2.73	39.48	
	0.2267	3.00	41.79	
Morphine hydrochloride ...	0.1395	3.37	75.24	75.91
	0.1395	3.38	75.46	
	0.1395	3.35	74.79	
Brucine hydrochloride ...	0.1261	2.47	84.37	84.29
	0.1261	2.48	84.71	
	0.0966	1.89	84.23	
Ephedrine sulphate	0.1556	5.05	58.52	58.86
	0.1556	4.98	57.70	
Quinine	0.1344	3.74	98.52	99.57
	0.1344	3.78	99.57	
Cinchonine	0.0976	3.14	98.46	98.15
	0.0976	3.13	98.15	

(b) DETERMINATION OF ALKALOIDAL CONTENT OF GALENICALS

The determination was carried out according to the same principles as for the alkaloidal salts. Tincture of ipecacuanha and tincture of nux vomica were adsorbed direct, while fluid extract of cinchona and tincture of opium had the weighed quantity diluted with 25 ml. of ethyl alcohol (96 per cent.). The further procedure was the same as that detailed above. The colouring matter is adsorbed in the column only in small measure, and the solutions of the alkaloidal bases obtained are coloured. The equivalence point must be determined electrometrically with the aid of the glass and calomel electrodes. With tinctures of nux vomica and ipecacuanha the potential change is, however, masked by the accom-

panying substances, but is none the less perceptible, provided that sensitive apparatus is used. Amounts corresponding to theory have been found in all experiments, except in the case of tincture of nux vomica

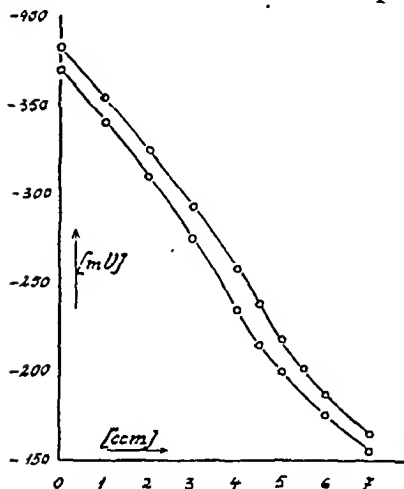


FIG. 1. Tincture of Ipecacuanha

when the result obtained was invariably double the theoretical figure. Figures 1, 2, 3 and 4 show the course of the titrations. In carrying out the titrations the ethyl alcohol solutions were diluted with water to 100 ml. In working the fluid extract of cinchona much depends upon the careful washing out of the adsorbed column, since cinchonine is far less soluble than quinine. In the case of opium a larger alkaloidal content was found that would correspond to morphine alone since the total content of morphine and other alkaloids was determined. The results are shown in Table II.

SUMMARY

1. A method has been worked out for the determination of salts of alkaloids by an adsorption method involving exchange of ions, on the synthetic resin known as Amberlite IR-4B which was used with the substances specified in Table I.

2. It is shown that this method is also suitable for determining the alkaloidal content of the galenicals specified in Table II.

TABLE II
DETERMINATIONS OF ALKALOIDS IN GALENICALS

Preparation Used	Weighed Out	N/50 Hydrochloric Acid ($f=1.092$)	Found by Adsorption Method	Found by Czechoslovak Pharmacopœia Method
	g.	ml.	per cent.	per cent.
Tincture of Ipecacuanha ...	48.80	4.00	0.107	0.095
	48.80	4.50	0.120	
	48.80	4.25	0.114	
	30.00	3.50	0.109	
Fluid Extract of Cinchona...	0.9452	2.65	4.733	4.450
	0.9756	2.65	4.586	
	0.9692	2.65	4.586	
	0.8315	2.25	4.568	
Tincture of Opium ...	2.9170	3.45	1.842	1.11
	1.3434	1.65	1.912	
	1.9841	2.20	1.727	
Tincture of Nux Vomica ...	35.27	10.00	0.563	0.247
	35.27	9.40	0.530	
	30.00	7.00	0.464	

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3. The advantage of the method is its rapidity, simplicity and the use of only small quantities of solvents, together with the determination of all the alkaloids present without any of the possibilities of loss arising from more complicated experimental procedures.

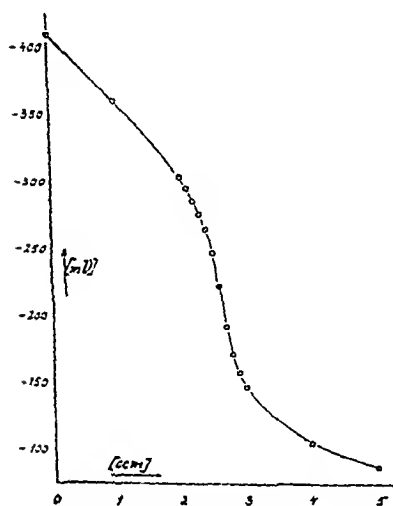


FIG. 2. Fluid Extract of Cinchona

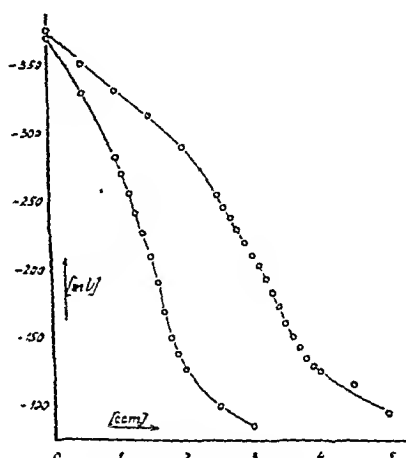


FIG. 3. Tincture of Opium

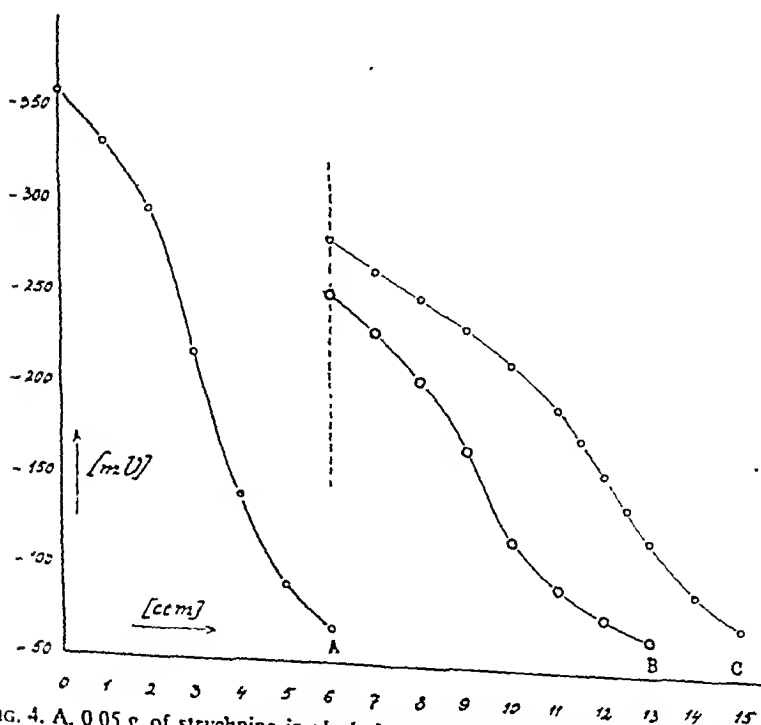


FIG. 4. A. 0.05 g. of strychnine in alcohol. B. Tincture of nux vomica. C. 0.05 g. of strychnine in tincture of nux vomica.

panying substances, but is none the less perceptible, provided that sensitive apparatus is used. Amounts corresponding to theory have been found in all experiments, except in the case of tincture of nux vomica

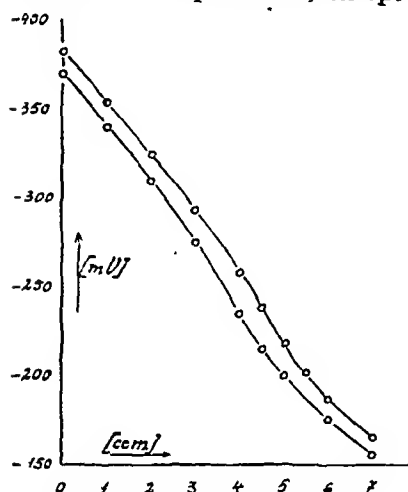


FIG. 1. Tincture of Ipecacuanha

when the result obtained was invariably double the theoretical figure. Figures 1, 2, 3 and 4 show the course of the titrations. In carrying out the titrations the ethyl alcohol solutions were diluted with water to 100 ml. In working the fluid extract of cinchona much depends upon the careful washing out of the adsorbed column, since cinchonine is far less soluble than quinine. In the case of opium a larger alkaloidal content was found that would correspond to morphine alone since the total content of morphine and other alkaloids was determined. The results are shown in Table II.

SUMMARY

1. A method has been worked out for the determination of salts of alkaloids by an adsorption method involving exchange of ions, on the synthetic resin known as Amberlite IR-4B which was used with the substances specified in Table I.

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TABLE II
DETERMINATIONS OF ALKALOIDS IN GALENICALS

Preparation Used	Weighed Out	N/50 Hydrochloric Acid (f=1.092)	Found by Adsorption Method	Found by Czechoslovak Pharmacopoeia Method
	g.	ml.	per cent.	per cent.
Tincture of Ipecacuanha ...	48.80 48.80 48.80 30.00	4.00 4.50 4.25 3.50	0.107 0.120 0.114 0.109	0.095
Fluid Extract of Cinchona...	0.9452 0.9756 0.9692 0.8315	2.65 2.65 2.65 2.25	4.733 4.586 4.586 4.568	4.450
Tincture of Opium ...	2.9170 1.3434 1.9841	3.45 1.65 2.20	1.842 1.912 1.727	1.11
Tincture of Nux Vomica ...	35.27 35.27 30.00	10.00 9.40 7.00	0.563 0.530 0.464	0.247

THE EFFECT OF DRUGS ON THE MOTILITY OF ISOLATED STRIPS OF HUMAN STOMACH MUSCLE

By J. D. P. GRAHAM

From the Department of Materia Medica, University of Glasgow

Received October 6, 1948

THE STRUCTURE and nerve supply of the human stomach, the movements of its muscle under a variety of conditions in health and disease, and the effect of various drug substances on gastric activity have been the subject of much research. Carlson¹ and Danielopolu² have written extensively on the subject and McSwiney³ reviews the literature widely. The majority of investigators have made use of a method which records the changes in pressure within balloons placed in the oesophagus, antrum, fundus, pylorus or duodenum of animals or man, or have attempted to assess the alterations in gastric motility by observation and photography with the aid of X-rays. The preparations were all in the fasting state; in some cases contractions of the stomach muscle were stimulated by the previous administration of insulin. Animals might or might not be under the influence of a variety of anæsthetics, or be subjected to ablation of varying portions of the central nervous system. Sundry nerves have been cut and stimulated or allowed to degenerate. From all this work much information has accrued but there has also arisen controversy as to the precise action of a number of drugs of therapeutic importance. One source of error may have arisen from misinterpretation of viscerographic records where the balloon has recorded the activity of the pyloric sphincter when it was supposed to lie in the body of the stomach, or where interpretation of roentgenological observations has been unwittingly influenced by subjective factors during visualisation on the screen.

Apart from the reports of Smith⁴ and of Tezner and Turolt⁵ very little work has been done with isolated strips of muscle from the human stomach wall, though innumerable experiments have been done with isolated pieces of intestine from animals. It would appear desirable to record the effects of various drugs on isolated portions of gastric muscle before proceeding to investigate and interpret the more complex pictures seen in intact animals and in man. With the recent spread of the practice of partial gastrectomy in cases of peptic ulceration it is now easy to obtain fresh samples of human stomach for investigation.

METHOD

Freshly prepared ice-cold Tyrode solution (sodium chloride 0.8 per cent., potassium chloride 0.021 per cent., calcium chloride (anhydrous) 0.02 per cent., magnesium chloride (anhydrous) 0.001 per cent., dextrose 0.1 per cent., sodium acid phosphate (anhydrous) 0.005 per cent., sodium bicarbonate 0.1 per cent.) was brought into the theatre during operation. As soon as possible after removal of the specimen from the patient, a healthy area of stomach was selected as far as

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it is seen that the essential characteristics of all the types of wave contraction described by clinical investigators as occurring in the intact stomach of man are to be found in the muscle strip with its absence of circulation, its absence of connection to the central nervous system and its relatively constant environment in a bath of nutrient fluid. It is not suggested that muscular contraction in the normal intact stomach is independent of vascular and nervous influence, but that all the elements for the various types of contraction recorded are available in the muscle and are capable of being carried on by the muscle acting independently. The initiation and regulation of the particular type of contraction found at any one time may well be under the influence of a wide variety of extraneous circumstances.

THE ACTION OF DRUGS

(a) *Parasympathomimetic compounds*.—Strips of isolated human stomach muscle do not respond to parasympathomimetic drugs in the same low concentrations as do isolated segments of gut from rabbits, guinea-pigs, etc. Acetylcholine in a concentration of 10^{-8} produces only a slight increase in motor activity, but a concentration of 10^{-7} gives rise to a much greater response. If the muscle is in a resting state (phase of relative quiescence) it contracts violently and may pass into a tetanic

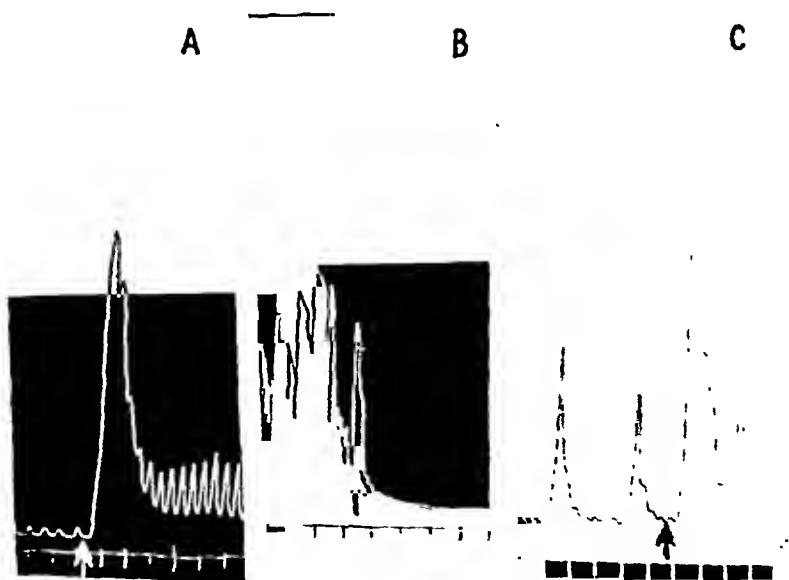


FIG. 1.—Record of movements from a strip of longitudinal muscle from the anterior wall of the human stomach, mounted at 37.5°C . in oxygenated Tyrode solution. Time in 30 sec. 1A shows the phase of relative quiescence changed into a marked motor response and a tetanic spasm by acetylcholine 10^{-7} . 1B shows the phase of active contractions inhibited by adrenaline 10^{-7} . 1C shows phase of active contractions (less violent than in 1B) stimulated by histamine (base) 10^{-6} .

possible from the diseased area and a portion of stomach wall some 5 cm. square cut off. The mucosa was separated from the muscle layers and the latter conveyed to the laboratory at once in fresh cold Tyrode solution. There a suitable piece of muscle approximately 2 cm. by $1/3$ cm. was cut in the long axis of the muscle fibres which is clearly marked, and mounted in the usual isolated organ bath in oxygenated Tyrode solution at 37.5°C . attached to a frontal writing lever. Preparations so mounted showed spontaneous activity of varying degree for 8 to 10 hours; portions preserved at 4°C . could be revived after 24 hours. Drugs were added in solution to make various final concentrations in the 75-ml. bath used. Between tests the preparation was washed twice with Tyrode solution and allowed 15 minutes or more to recover.

MATERIAL

The specimens used in this work were from 8 cases of chronic duodenal ulcer operated upon for intractable pain. The muscle fibres used for test were taken from the anterior or posterior wall of the stomach as far from the pyloric region as possible, i.e., the reactions to be described are those of the longitudinal muscle of the body of the human stomach. The stomachs were free from disease in themselves as there was no gastric ulcer or inflammation and no pyloric stenosis or gastric atony or stasis.

THE NORMAL CONTRACTION

Anderson⁶ using a viscerographic method describes a phase of relative quiescence characterised by small flat topped waves of about $2\frac{1}{2}$ minutes duration, a phase of active contractions and a phase of tonus waves seen in the course of spontaneous gastric activity and a tetanic phase seen in special circumstances. In the present work all these types of contraction were noted. The tetanic phase only occurs under the influence of an abnormal stimulus such as the addition of a parasympathomimetic drug (see Fig. 1A). The phase of quiescence is the usual finding after the preparation is first mounted and also occurs spontaneously for long and variable periods. It is characterised in the muscle strip by the occurrence of small irregular contractions of some 10 to 20 sec. duration with a return to the base line between each contraction (see Fig. 1A). A modified form of this activity may be found to occur during the period of relaxation between the powerful contractions of the phase of active or hunger contractions (see Fig. 4B) though this is not always the case (see Fig. 2B). The phase of tonus contractions is often found and is characterised by flat-topped contractions of about 2 minutes duration, with variable degrees of relaxation in the course of the wave plateau but no return to the base line (see Fig. 4A). The active or hunger contraction is the most characteristic activity and occurs in spontaneous bursts which reach a maximum degree of contraction after some 10 contractions, continue for a variable period from 5 minutes to 2 hours or more, and cease abruptly or more commonly by declining in vigour to assume the characteristics of the tonus wave or the phase of relative quiescence. Thus

the effect of histamine 10^{-6} . Barium chloride 10^{-3} also causes increased motor activity of an irregular type and may give rise to tetany.

(d) *Spasmolytic compounds*.—The action of atropine sulphate on this preparation was examined in detail because of the controversy as to whether this drug produces a motor response in certain small doses, as Danielopolu² and Anderson and Morris⁷ suggest, or is invariably inhibitor in action as Henderson and Sweeten⁸ maintain. In the majority of specimens atropine sulphate produced an inhibitor response in all concentrations of the drug which showed any action. If the muscle was in a phase of active contraction the contractions were diminished in extent though they might become rather more frequent. The inhibitory action of atropine in a concentration of 5×10^{-6} on a muscle strip in the phase of active contraction is shown in Figure 2A. Lesser concentrations than 10^{-7} had no effect, and usually 10^{-6} was needed to produce any great degree of inhibition. The activity termed the phase of tonus waves was likewise inhibited by atropine and the action of acetylcholine prevented or abolished. If the muscle was in a phase of relative quiescence the small movements diminished or disappeared.

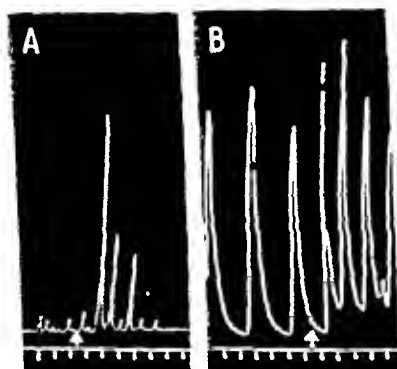


FIG. 3.—As in Figure 1. 3A shows interruption of a long period of relative quiescence by a short burst of active contractions after adding atropine sulphate 10^{-7} . 3B shows the increase in tone and rate of contraction caused by adding atropine sulphate 10^{-7} during phase of active contractions.

In 3 specimens out of the 8 examined atropine sulphate in a concentration of 10^{-7} produced a motor response. This consisted of either a speeding up of the contractions seen in a phase of active contraction (see Figure 3B) with a slight rise in tone, or the production of a short burst of active contractions in the middle of a prolonged period of relative quiescence (see Figure 3A). Further addition of atropine caused inhibition of the stomach muscle, but the effect could be repeated after a period of absence of drug of $\frac{1}{2}$ to 1 hour.

Pavatrine (diethylaminoethyl fluorene carboxylate), which has been shown by Lehmann and Knoefel⁹ to have less than $1/20$ of the potency of atropine in reducing the hypermotility caused in the

stomach of the anaesthetised dog by previous injection of insulin, was active as an inhibitor of contractions in the muscle strip. The concentration required to produce an effect similar to that of atropine 5×10^{-6} was about 10^{-5} (see Fig. 2B). In the small number of tests made on isolated human gastric muscle it would therefore appear that pavatrine approximates more nearly to the activity of atropine than the work of Lehmann and Knoefel⁹ on anaesthetised insulin-injected dogs would suggest.

THE MOTILITY OF HUMAN STOMACH

gastric muscle by adrenaline is in agreement with Smith⁴, Tezner and Turolt⁵, Dickson and Wilson¹⁴, Barron¹² and Anderson and Morris⁷. It would appear that adrenaline and acetylcholine and their synthetic analogues act in a similar manner upon the isolated gastric muscle as upon the intact stomach in man and in animals. Since histamine is used clinically to promote a flow of gastric juice its action on stomach movements is of some importance. According to Schenk¹³ it increases gastric motility (roentgenological studies); according to Anderson and Morris⁷ (viscerographic studies) it inhibits gastric activity. Histamine has a motor effect upon a wide variety of isolated preparations of smooth muscle, but not on all. Nevertheless it is unusual for the action to vary in any one species between *in vitro* and *in vivo* preparations of the same type of muscle, especially in the absence of anaesthesia. The dose used by Anderson and Morris⁷ was 1.0 mg. per patient, which was less than that used by Schenk¹³, who gave 6 to 8 mg. per patient. The latter dose may approximate more closely to that used in the present work (10^{-6} concentration). Both X-ray and viscerographic recording in intact human beings have certain weaknesses, not least of which is the possible introduction of autonomic activity in the patient following upon psychic disturbance as a result of hypodermic injection.

The action of atropine and other spasmolytic compounds is mainly that of inhibition of gastric motility as Bastedo¹⁵ and Henderson and Sweeten⁸ claim, but the finding of Danielopolu² and Anderson and Morris⁷ that small doses of atropine may increase gastric activity is also supported. The nature of this activity is obscure. The differing effects of morphine found with the isolated muscle may help to explain the confusing effects reported by Tolley and Abbot¹⁶, Myers¹¹ and Anderson and Morris⁷.

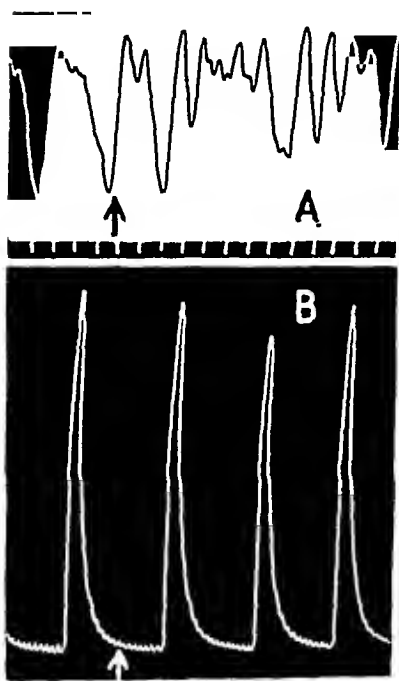
The general conclusion reached was that the actions of the drugs in common use described were essentially the same in intact patients and in isolated portions of stomach muscle and that the differences observed could probably be explained by complexities in interpretation of recordings from the intact human being, and complexities due to modification of the action of the drugs by influences from the nervous system of the patient (psychogenic and otherwise), influences from the varying content of the viscera, and influences from varying doses and routes of administration of the drugs in clinical use. The beneficial effect of belladonna in the treatment of peptic ulceration may well be explained by the sedative effect of adequate doses of atropine on gastric motility and the blocking effect of this drug on the part played by vagal activity in gastric secretion.

SUMMARY

1. Strips of longitudinal muscle were obtained from the anterior and posterior wall of the human stomach removed at gastrectomy for duodenal ulceration. The preparations were mounted in an isolated organ bath and movements recorded.

(e) *Anti-histamine Compounds*.—Recently Graham¹⁰ compared the spasmolytic potency of benadryl, neoantergan (2786RP) and antistine. In view of the property displayed by these compounds of inhibiting contractions of gut muscle caused by barium and acetylcholine as well as histamine it is not surprising that a concentration of 10^{-5} neoantergan or of benadryl reduces the extent of the active contraction and the tonus wave of gastric muscle (see Fig. 2C). Benadryl is less powerful than neoantergan in abolishing spasm induced by the addition of histamine but is the more active of the two in inhibiting spontaneous contractions of the gastric muscle.

(f) *Morphine*.—The action of morphine has been the subject of much discussion. Myers¹¹ working with a viscerographic record of decerebrate cats reported mostly an inhibition of gastric muscle activity with occasional responses by increased movement after injection of morphine. Anderson and Morris⁷ working on human subjects reported a diminution of gastric movement followed by a temporary increase and a final state of relative quiescence in fasting patients showing a phase of active contraction.



With isolated strips in the present work varying effects were obtained. The quiescent muscle and the actively contracting muscle were not affected by morphine tartrate in concentration from 10^{-7} to 10^{-5} (see Fig. 4B). The muscle in tonus waves showed an increase in tone with concentration from 10^{-6} to 10^{-4} . No tetanic spasm was produced nor did the amplitude of the individual wave contractions increase (see Fig. 4A).

DISCUSSION

The statement that the fasting stomach is never entirely at rest is supported by the observation of activity of some sort in isolated strips of human gastric muscle.

Hollow smooth muscle viscera with a variable content are seldom at rest. The stimulant action of parasympathomimetic compounds found in this work supports the findings of Smith⁴ and Barron¹², and that of histamine agrees with the findings of Schenk¹³ while disagreeing with Anderson and Morris⁷. The inhibition of isolated

FIG. 4.—As in Figure 1, 4A shows increase of tone during phase of tonus wave changes after adding morphine tartrate 10^{-6} . 4B shows the absence of effect after adding morphine 10^{-5} during the phase of active contractions.

THE EVALUATION OF TRAGACANTH BY MEANS OF THE APPARENT VISCOSITY DETERMINED IN A STANDARD U-TUBE VISCOMETER

PART II.—WHOLE GUM

By W. P. CHAMBERS

From the Laboratory of Damancy & Co., Ltd.

Received August 25, 1948

IN A PREVIOUS COMMUNICATION¹ a method was suggested for the routine comparison of the apparent viscosities of powdered tragacanth by the use of a U-tube viscometer.

It was found that in order to use this type of instrument the sample of mucilage must be relatively homogeneous, a condition that was arrived at by the combined application of heat treatment and mechanical homogenisation. Using the U-tube viscometer it was not found possible to attain homogeneity by heat treatment alone without reducing the viscosity of the sample to figures that bore but little relation to the original viscosity; and as the object was to exhibit the gum at a point as near as possible to the maximum viscosity, both heat and mechanical treatment were found to be necessary. The problems involved in the evaluation of the whole gum are essentially those encountered in the powder, but modified to some extent by the intractable nature of the flake.

EXPERIMENTAL

An examination of a number of commercial samples of whole gum showed great diversity in size, thickness, texture and colour; while attempts to prepare mucilages from flake, without preliminary treatment and within those limits which are known to be without deleterious action on the viscosity, resulted in complete failure. That mucilages can be prepared from whole gum by treatment with boiling water or by immersion in a water-bath over a sufficiently long period is, of course, recognised, but as previous experiments show, such a method of ensuring homogeneity does not yield comparable results when determinations are carried out in a U-tube viscometer. It has long been recognised that the quality of tragacanth is adversely affected when the whole gum is reduced to powder by the customary commercial methods, and this has usually been thought to be due to mechanical destruction or to the heat created during grinding; but whatever the cause, it is advisable that both these attributes to a reduction of viscosity be maintained at the lowest level.

The method as finally adopted consists of "kibbling" in a mortar a suitable quantity of a representative sample of the flake until it passes a No. 30 sieve, after which the sample is thoroughly mixed. Attempts at preparing mucilages from this treated material, although an advance over the whole gum, resulted in products which, even after standing

2. Spontaneous movement included periods of relative quiescence, periods of slow wave changes in tonus, and periods of active contraction. Periods of tetanic spasm could be induced by drugs.

3. Acetylcholine, carbaminoylcholine, eserine, barium and histamine stimulated the muscle, and adrenaline, ephedrine and amphetamine inhibited it.

4. Atropine, pavatrine, benadryl and neoantergan (2786 RP) inhibited spontaneous and drug induced contractions. Small doses of atropine (concentration of 10^{-7}) occasionally had a motor effect on the muscle strip.

5. Morphine had no effect on the quiescent or actively contracting muscle, but increased the tone of the slow wave of tonus change. Irregularity of spontaneous activity and response to drugs was a marked feature of the preparation.

This work was done during the tenure of an I.C.I. Fellowship in Pharmacology. It is desired to thank Prof. C. F. W. Illingworth and Mr. R. A. Jamieson, of the Peptic Ulcer clinic at the Western Infirmary, Glasgow, for the specimens of human stomach.

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THE EVALUATION OF TRAGACANTH—PART II

is thereby accelerated to an approximate maximum and at the same time such temperature is without undue significance in its destructive effect on the viscosity.

As the strengths of the mucilages made from whole gum were the same as those previously used in experiments on the powder, namely, 0.5 per cent., it was at once apparent in the samples so far examined that the viscosities obtained when using whole gum were far higher than those encountered in the case of the powdered drug. Using a No. 3 U-tube viscometer ($K = 0.306$) it is unusual, at a strength of 0.5 per cent., to find times of flow for the powdered gum in excess of 1000 sec., yet in the case of whole gum times of the order of 2000 to 5000 sec. are quite common.

It would thus appear that the whole gum is a far superior article, as judged by apparent viscosity, than the generally available commercial powder. The knowledge that the gum yields a product of inferior quality when ground leads naturally to the assumption of different standards for the two forms; and this fact renders the use of the U-tube viscometer more rational than otherwise would be the case. In Table II are recorded a few results showing the figures for times of flow of mucilage of whole gum, when partly ground and when fully ground, and although the complete history of the gums was not fully known, the figures nevertheless suggest that a reduction of viscosity may be expected on grinding.

TABLE II

THE EFFECT OF GRINDING ON THE VISCOSITY (TIMES OF FLOW IN SEC.) OF 0.5 PER CENT. HOMOGENEOUS MUCILAGES PREPARED FROM WHOLE GUM, PARTLY GROUND AND FULLY GROUND GUM

Sample						Whole Gum	No. 80 Powder	No. 140 Powder
1	1657	1302	840
2	300	205	121
3	167	172	98
4	2830	—	1830
5	2760	—	1962
6	286	—	183

Strengths of mucilage that are applicable to the powder are, when applied to the whole gum, found to yield times of flow that are altogether too time-consuming for routine comparisons, and it is necessary to reduce the strength of the flake to yield figures that are comparable to those obtained when using the powder. A reduction in weight from 1.0 g. to 0.75 g. results in times of flow for the flake which are of the order of those obtained for the powder. The effect of reducing the strength of the mucilage is seen to be roughly proportional over the range of concentrations examined (Table III).

Method.—Kibble a representative sample of whole gum in a mortar until it passes a No. 30 sieve. To 0.75 g., accurately weighed, contained in a 300-ml. conical flask, add 5 ml. of alcohol (95 per cent.) followed

for several days, showed large aggregations of undispersed gum. The problem of effecting "solution" of the gum was finally solved by the use of a mechanical stirrer. A simple Pyrex all-glass stirrer operated by compressed air was found to be very satisfactory. The addition of alcohol prior to the addition of water serves to keep the particles of gum sufficiently separated until the stirrer can be introduced into the flask. In the absence of alcohol, aggregates of gum are formed which are not usually dispersed by subsequent stirring. Fairly good dispersion is usually effected in less than one hour's stirring.

It soon became obvious that the employment of the same technique as that used for the powder, namely, allowing to stand at room temperature for 48 hours, would not result in full hydration of the gum. In addition, most samples contained a proportion of woody debris in amounts that would probably block the jet of the homogeniser. The mucilage, therefore, after standing for about 24 hours, was "cleaned" by passing it twice through a No. 100 sieve by means of reduced pressure. It was usually necessary to assist the mucilage through the sieve by means of a very small stencil brush, finally removing any mucilage adherent to the under surface of the sieve by means of a spatula. For this purpose it is desirable to use a piece of apparatus which can easily be dismantled and a suitable combination may be made from a Phoenix filter funnel, diameter $3\frac{1}{2}$ inches, and a Sifting Investigator outfit carrying B.S.S. sieves of $3\frac{7}{8}$ inches diameter. With a view to accelerating the rate of hydration and studying the effect of maintaining mucilages at different temperatures, a series of samples were stored in an oven operating at controlled temperatures. Table I shows the effect of such treatment on four different gums.

TABLE I

THE EFFECT ON THE VISCOSITY (TIMES OF FLOW IN SEC.) OF 0.5 PER CENT. MUCILAGES, PREPARED FROM WHOLE GUM, OF MAINTAINING THEM AT VARIOUS TEMPERATURES

Period	48 hours	24 hours	48 hours	48 hours	48 hours	48 hours
Temperature °C.	Room	40°	40°	50°	60°	70°
1	243, 283 258	272, 260 342	351, 355, 374 422, 367, 361	447, 451, 450 478, 439, 430	421, 408, 412 418, 423, 439	189, 246, 171 237, 210
2	2500	2734	2520, 3022	3300, 2820	1805, 1735	925
3	3840	3720	5015, 4800	3670, 4080	2440, 2930	813
4	1735	2160	2280, 2290	2100, 2040	1680, 1765	814

From these experiments it emerges that, in general, using a U-tube viscometer, maximum hydration is not attained by allowing the mucilage to stand at room temperature for 48 hours. The viscosity is seen to rise until a maximum is reached around 40°C., above which a falling off occurs, while a temperature of 70°C. has a very marked effect in reducing the viscosity. It may reasonably be assumed, therefore, that in employing an oven temperature of 40°C. for 48 hours, the hydration

THE EVALUATION OF TRAGACANTH—PART II

Results of viscosity determinations on whole gum are seen to be considerably more variable than those on the powdered drug, and this is thought to be caused by a more variable rate of hydration resulting from the use of a kibbled product, which presents a surface to the hydrating medium that is only a fraction of that presented by a fairly uniform fine powder; in addition to the methodic errors the operative errors are also greater.

SUMMARY

1. A method is described for the routine comparison of the apparent viscosity of whole gum.

2. When determined in a U-tube viscometer, mucilages prepared from whole gum reach maximum hydration, and therefore maximum viscosity, after storage for 48 hours at a temperature of 40°C. With shorter periods at lower temperatures hydration is not complete and the viscosity is below the maximum, while with higher temperatures over the same period, although hydration is complete, the viscosity is reduced by virtue of the destructive effect of the higher temperatures.

3. At the same concentration, mucilages of whole gum yield higher viscosities than when commercially powdered, thereby indicating a loss of quality mainly due to grinding.

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REFERENCE

1. Chambers, *Quart. J. Pharm. Pharmacol.*, 1948, 21, 44.

by 200 ml. of water, swirling the contents of the flask during the addition. Immerse the flask up to the neck in a water-bath maintained at from 40° to 50°C., insert a suitable mechanical stirrer and stir at a speed sufficient to maintain movement of the particles for about one hour. Remove from the bath, stopper the flask and place for a period

TABLE III

THE EFFECT OF VARIATION IN THE PERCENTAGE STRENGTHS OF GUM ON THE VISCOSITY (TIMES OF FLOW IN SEC.)

Concentration	0.25 per cent.	0.33 per cent.	0.375 per cent.	0.50 per cent.	Ratios
1	163	455	694	3000	1 : 2.8 : 4.3 : 18.4
2	165	460	838	3700	1 : 2.8 : 5.1 : 22.6
3	129	296	525	2300	1 : 2.3 : 4.1 : 17.8

of 24 hours in an oven maintained at 40°C. Attach a reflux condenser and immerse the flask up to the neck in a bath of boiling water for 5 minutes, swirling the contents of the flask vigorously for 5 seconds at the end of the first, second, third and fourth minutes. Remove from the source of heat and allow to cool to about 40°C. with occasional shaking and replace in the oven for 3 or 4 hours. Remove from the oven and by means of reduced pressure pass the contents of the flask as completely as possible through a No. 100 sieve twice. Replace in the oven until about 48 hours have elapsed from the time of commencement of the assay, after which pass the mucilage 3 times through a Q.P. homogeniser using a uniform and even pumping speed of about one complete stroke per sec. Determine the apparent viscosity (time of flow in seconds) at 20°C. in a No. 3 B.S. U-tube viscometer. The results of the application of this method to a number of samples of flake tragacanth are recorded in Table IV.

TABLE IV

THE RESULTS OF THE EVALUATION OF WHOLE GUM SAMPLES BY DETERMINATION OF THE APPARENT VISCOSITY (TIMES OF FLOW IN SEC.) IN A NO. 3 B.S. U-TUBE VISCOMETER

Sample	A	B	C	D	E	F	G	H	I
	637	709	45.0	126	377	572	740	145	657
	661	645	44.0	132	370	600	805	143	674
	665	690	46.0	129	382	579	827	142	702
	623	660	45.2	126	373	566	785	142	730
	643	675	45.5	131	350	576	720	144	712
	657	730	45.1	120	390	586	790	141	700
Mean	648	685	45.1	127	374	580	778	143	696
Standard deviation per cent.	± 2.5	± 4.6	± 1.47	± 3.4	± 3.7	± 2.1	± 5.2	± 1.1	± 3.8

1 inch, 50 times at intervals of 2 seconds. The powder then occupies a volume V_a , which is reproducible to within ± 1 ml. in 150 ml., giving an apparent density (W/V_a) variation of ± 0.002 g./ml., using apparatus of specified dimensions. The dropping interval of 2 seconds is the most rapid procedure that can conveniently be carried out. There is no advantage in using a sieve finer than 30-mesh, and there will be a greater tendency for shearing to alter the apparent density. Increasing the height or number of times the cylinder is dropped lessens the difference in V_a for different grades of powder. The test can be modified for use with other powders, such as light magnesium carbonate, diatomaceous earth, chalk and barium carbonate. G. B.

Jaborandi, Assay of. Report No. 5 of the Poisons Sub-Committee of the Analytical Methods Committee of the Society of Public Analysts. (*Analyst*, 1948, 73, 311.) In the method recommended, powdered jaborandi leaves made alkaline with dilute solution of ammonia, are extracted by percolation with chloroform; this is continued until complete extraction is effected as shown by a test with Mayer's reagent. The alkaloids are extracted from the bulked chloroform solutions using 0.1N sulphuric acid and the resulting acid extract, after making alkaline with ammonia, is finally extracted with successive quantities of chloroform. The chloroform extracts are combined, evaporated, dried, dissolved in a standard excess of 0.05N sulphuric acid and titrated with 0.05N sodium hydroxide. The result obtained expresses the total alkaloid content calculated as pilocarpine. Details of the extraction procedure and a method for separating pilocarpine nitrate from the total alkaloids are given. R. E. S.

Morphine and Apomorphine, Determination of, by a Volumetric-Colorimetric Method. A. Ionesco-Matiu, J. Popa and L. Monciu. (*Ann. pharm. Franc.*, 1948, 6, 25.) In a 200-ml. conical flask, place a known volume of the morphine solution, 0.5 to 2 ml., and evaporate to dryness on a water-bath; to the residue add 2 ml. of concentrated sulphuric acid, and heat to boiling on a water-bath for 30 minutes; cool, and add with shaking, 100 ml. of distilled water, and neutralise to sodium, using one drop of phenolphthalein solution. Add to the solution 5 drops of a saturated solution of corrosive sublimate and 5 drops of a 10 per cent. solution of sodium acetate; heat to boiling for one minute, when the solution changes from colourless to green, through violet to an intense blue. Cool the solution under a current of water, add 2 ml. of 20 per cent. sulphuric acid and, using a micro-burette, add drop by drop solution of N/10 potassium permanganate, until the solution has changed to a yellowish-brown colour, the permanganate solution maintaining its colour for several seconds; each ml. of N/10 potassium permanganate is equivalent to 0.00495 g. of morphine hydrochloride. The same method may be employed for the determination of apomorphine, commencing with the words "Add to the solution 5 drops . . ."; each ml. of N/10 potassium permanganate is equivalent to 0.00294 g. of apomorphine hydrochloride. The determination of either morphine or apomorphine may be made even in the presence of other alkaloids. S. L. W.

Plant Extracts, Identification of. G. di Bacco. (*Boll. chim-farm.*, 1948, 87, 124.) Extracts of medicinal plants can be identified and their strength and purity established by chromatographic tests. Aluminium oxide is used as the medium, in a glass tube 15 mm. in diameter and 180 mm. off to a point so that the liquid can flow out at the rate of

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

l-nor-Ecgonine, Complete Extraction of the Bases and Acid Esters from Coca Leaves. A. W. K. de Jong. (*Rec. Trav. chim. Pays-Bas*, 1948, 67, 153.) A considerable quantity of a number of bases has been extracted for quantitative determinations, from coca leaves at 55°C. with a mixture of benzene, methyl alcohol and N/1 ammonium hydroxide, but some of these are not ecgonine alkaloids. Since the ecgonine alkaloids are soluble in ether, the benzene and methyl alcohol extraction was replaced by ether extraction in a Soxhlet apparatus, but this method too had its disadvantages. When it was proved that coca leaves contain *l-nor*-ecgonine, which could be converted by methylation into *l*-ecgonine, and thus was of value for preparing cocaine, a return to the benzene and methyl alcohol extraction at ordinary temperature, to prevent decomposition of the methyl ester, was indicated. A smaller quantity of the bases was extracted at ordinary temperatures than at 55°C. and it was necessary to add water to the menstruum, care being taken not to add more than was required to dissolve the salts of the bases. For the extraction now proposed, coca leaves, 20 g., are mixed with finely powdered calcium oxide, which rapidly absorbs all the water contained in the leaf tissue, and dried for 24 hours. They are then shaken with 100 ml. of anhydrous benzene, 5 ml. of absolute methyl alcohol, and a volume of ammonia solution, containing exactly 1.938 g. of water. After another 24 hours, a quantity of finely powdered calcium oxide, corresponding to the amount of water used in the ammonia solution, is added, and after thoroughly mixing, the extractor is closed and the mixture allowed to stand. The calcium oxide absorbs the water and the calcium hydroxide formed sets free the ammonia and the bases, while the acid esters of *l-nor*-ecgonine are converted into their calcium salts; decomposition of the alkaloids is thus prevented. Percolation is started after 24 hours at a slow rate (about 5 drops per minute), and when the solvent mixture has been used, a quantity of a mixture of anhydrous benzene and absolute methyl alcohol, which boils at 58°C., is added to the leaves. The percolation is stopped when 800 ml. of percolate has been collected, and the benzene-methyl alcohol mixture distilled at 58°C. The remaining benzene is filtered from the calcium salts, which are insoluble in benzene. The residue is shaken with 25 ml. of N/5 hydrochloric acid, and 100 ml. of ether and the quantitative determination of the bases (by titration, using methyl red solution as indicator), of the cocaines (using N/1 sodium carbonate), and finally of *l-nor*-ecgonine is carried out on the hydrochloric acid layer.

L. H. P

ANALYTICAL

Apparent Density of Dry Powders, a Method for the Determination of. W. B. Ault. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 313.) 40 g. of precipitated calcium carbonate is rubbed through a 30-mesh sieve and transferred, without jolting or shaking, to a 250 ml. glass measuring cylinder. The powder occupies a volume V_0 , depending upon its lightness or bulkiness, but it is difficult to obtain reproducible results for this quantity. The cylinder is fitted with a bung, placed in a box and allowed to drop through

1 inch, 50 times at intervals of 2 seconds. The powder then occupies a volume V_d , which is reproducible to within ± 1 ml. in 150 ml., giving an apparent density (W/V_d) variation of ± 0.002 g./ml., using apparatus of specified dimensions. The dropping interval of 2 seconds is the most rapid procedure that can conveniently be carried out. There is no advantage in using a sieve finer than 30-mesh, and there will be a greater tendency for shearing to alter the apparent density. Increasing the height or number of times the cylinder is dropped lessens the difference in V_d for different grades of powder. The test can be modified for use with other powders, such as light magnesium carbonate, diatomaceous earth, chalk and barium carbonate.

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S. L. W.

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30 drops a minute. The lower end of the tube is plugged with cotton wool and about 10 g. of aluminium oxide suspension in light petroleum is poured in; the column of oxide should occupy 90 mm. When the light petroleum has flowed out, leaving a layer 8 or 10 mm. above the oxide, the solution or suspension of the extract to be examined is poured on, allowed to pass through, and the chromatogram developed by washing with two lots of light petroleum. For liquid extracts of rhubarb, cascara and frangula, 1 g. of the extract is shaken from time to time during 30 minutes with light petroleum 10 ml., benzene 10 ml., ether 5 ml. and the clear supernatant liquid is poured on the oxide. The liquid extract of genuine rhubarb of the Italian Pharmacopœia gave a 2 mm. yellow band, an 8 mm. red band, a 10 mm. pink fringe; a liquid extract of genuine rhubarb soluble in syrup gave a 1 mm. yellow band, a 6 mm. red band, a 12 mm. pink fringe; a liquid extract of European rhapontic rhubarb gave a 0.5 mm. yellow band, a 12 mm. pink fringe; a mixture of equal parts of genuine and rhapontic liquid extracts gave a 0.5 mm. yellow band, a 3 mm. red band, a 12 mm. pink fringe. Evidently the red band is due to anthraquinone derivatives, and shows the rhapontic rhubarb to be inferior to the Chinese and the soluble extract inferior to the official. The liquid extract of cascara of the Italian Pharmacopœia gave a 0.5 mm. yellow band, a 1 mm. red band, a 3 mm. bright red band, a 16 mm. pink fringe (the column viewed from above is coloured yellow). The aromatic, bitterless extract of cascara of the Italian Pharmacopœia gave a similar chromatogram with a more accentuated yellow band; liquid extract of frangula gave a 2 mm. bright red band and a 4 mm. pale pink fringe. To verify an extract, the chromatogram should be compared with one obtained from an extract of proved authenticity and the activity may be judged to be proportional to the length of the coloured bands.

H. D

FIXED OILS, FATS AND WAXES

Whale Oil, Component Acids and Glycerides of. T. P. Hilditch and L. Maddison. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 253.) The component acids of Antarctic whale oil have been previously separated by means of their lithium and lead salts, with subsequent analysis by ester fractionation. They have now been re-examined by the more recent process of crystallisation from solvents at low temperatures. This method is easier and quicker and gives results agreeing with the earlier. The results are given below.

COMPONENT ACIDS OF ANTARCTIC WHALE OIL

	By lithium and lead salt separations	By low-temperature crystallisation	
		(a)	(b)
	per cent (wt)	per cent (wt)	per cent (wt)
Lauric	0.2	Trace	0.3
Myristic	9.3	9.2	9.3
Palmitic	15.6	15.6	15.6
Stearic	2.8	1.9	2.1
Arachidic	0.3	0.6	0.2
Unsaturated C ₁₄	2.5	2.5	2.6
" C ₁₆	14.4	13.9	13.8
" C ₁₈	35.2	37.2	36.9
" C ₂₀	13.6	12.0	12.2
" C ₂₂	5.9	7.1	6.8
" C ₂₄	0.2	—	—

CHEMISTRY—ANALYTICAL

The component glycerides have also been segregated by crystallisation from acetone at -60°C . upwards. The following figures are similar to previous results, though differing in some respects: about 16 per cent. of disaturated and 2.5 per cent. of trisaturated glycerides, about 30 per cent. of tri-unsaturated glycerides, and about 50 per cent. of glycerides containing one saturated acid, one unsaturated C_{18} acid, and one of the other homologous unsaturated acids. About 45 per cent. of the oil contains acids of the C_{20} and C_{22} series, and oleic groups are present in over 90 per cent. of the oil.

H. F.

INORGANIC

Sulphur Absorbed by Clays, Chemical Activity of. A. Malquori. (*Ann Chim. appl.*, Roma, 1948, 38, 146.) The chemical activity of the sulphur absorbed by clays when heated with them was tested by boiling a weight of the clay containing 0.5 g. of sulphur with 0.5 g. of calcium hydroxide and 100 ml. of water and boiling for exactly 1 minute, cooling rapidly away from air and determining the sulphur in 10 ml. by oxidation and conversion to barium sulphate. If these sulphurised clays are kept in a moist atmosphere the sulphur rapidly becomes insoluble in calcium hydroxide; the loss in solubility varies for different clays from 8 to 45 per cent. in 14 days. The author connects this behaviour with the hygroscopic water.

H. D.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Anti-pernicious Anæmia Factors from Liver, Purification of. E. Lester Smith. (*Nature*, 1948, 161, 638.) This is a report of the preparation from ox liver of two pigments, both highly active in pernicious anæmia. The crude extract was purified by the methods employed by Emery and Parker (*Biochem. J.*, 1946, 40, iv) and then by repeated chromatography; subsequently, proteolysed liver extract was used as the starting material and gave better yields of a product with a higher activity. Four tons of material yielded barely 1 g. of the red material from 6 separate lots of liver. The minimum effective single dose for the best 4 preparations from non-proteolysed liver was assessed at about 0.6 mg., proteolysed liver yielding 1 preparation (L.E.445) effective at 0.3 mg. Ten batches of red material all proved clinically active (in 26 cases), and it was found that activity and colour are inseparable. Further chromatographic purification of one of these preparations, following the action of mixed bacteria, gave a product with 8 times the colour intensity of L.E.445, which, if activity remains proportional to colour, should be effective at 0.04 mg. The products are amorphous solids, with the colour of cobalt salts, but showing only general absorption of visible and ultra-violet light, and with molecular weights, as indicated by the diffusion method, of about 3,000 for the pigments from both proteolysed and non-proteolysed liver. On exposure to daylight there is a gradual change in colour from red to orange, accompanied by a marked change in chromatographic behaviour. The products are exceedingly soluble in water, soluble in nearly anhydrous alcohol, acetone, and glacial acetic acid, but insoluble in ether, chloroform and non-polar solvents. The author concludes that the two pigments are differing forms of the classical liver fraction first postulated by Minot and Murphy, and are not an incomplete substitute, such as folic acid or thymine. True pernicious anæmia and its associated neurological disturbances do not require a multiplicity of factors but respond to a single

factor. With an effective dose equivalent to some 20 mg. (and possibly only 2.5 mg.) daily, this is one of the most potent of known physiologically active substances.

S. L. W.

Oestrogens, Preparation from Urine by Application of High Temperatures. Felix Sulman. (*Nature*, 1948, 161, 605.) The usual methods of extracting oestrogens from the urines of pregnant women, pregnant mares, or stallions, with solvents which are not miscible with water, have certain inherent disadvantages. Large extraction vessels and large volumes of urine are required and this involves using a large distillation apparatus and consequently high fuel consumption for evaporation of the solvent; in addition the oestrogens are usually contaminated with organic material. In overcoming these difficulties it was found that the urine could be evaporated till a sticky, gum-like residue remained; this was heated to a temperature not exceeding 245°C. for 5 minutes so as to carbonise the bulk of the organic matter. The oestrogens, such as oestradiol, oestrone, oestriol, hippulin, equilin and equilin were not destroyed by this procedure. The carbonised mass was extracted with organic solvents and the extract, containing the oestrogens in rather high concentration, was evaporated. The dry residue obtained possessed a high degree of purity, since most organic impurities had been carbonised, and was further purified by the usual methods.

L. H. P.

Stilbæstrol Monoglucuronide, Isolation from Human Urine. K. S. Dodgson and R. Tecwyn Williams. (*Nature*, 1948, 161, 604.) It has been claimed by Wilder Smith (*Nature*, 1947, 160, 787) that 50 per cent. of stilbæstrol administered to human beings is excreted as a monoglucuronide. The authors were able to isolate the benzylamine salt of stilbæstrol monoglucuronide in a pure crystalline state from the urine of two women, immediately after parturition. These patients had received a total of 100 mg. of stilbæstrol each in 24 hours. The benzylamine salt had m.pt. and mixed m.pt. 223°C. and was lævo-rotatory with $[\alpha]_D^{20} = -55^\circ$ ($c = 0.2$ in 50 per cent. aqueous acetone). It appeared identical with a sample previously prepared from pure stilbæstrol monoglucuronide isolated from rabbit urine. The yield of the benzylamine salt corresponded to 35 per cent. of the stilbæstrol which had been administered. In addition to the monoglucuronide, small amounts of free stilbæstrol were also detected in these urines.

L. H. P.

***o*-Thymotinic Acid, Preparation and Inhibitory Properties of Derivatives.** J. P. Street, C. E. Georgi and P. J. Jannke. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 180.) *o*-Thymotinic acid (1-methyl-2-carboxy-3-hydroxy-4-isopropylbenzene) is structurally related to thymol and salicylic acid. Prepared by treating a solution of thymol in boiling xylene with metallic sodium and dry carbon dioxide at atmospheric pressure, it was obtained in a yield of 71.1 per cent, as colourless, needle-like crystals, m.pt. 126°C., soluble in organic solvents. The preparation of the mono- and di-sodium, silver, magnesium, calcium, barium, mercuric, zinc, cupric, lead, ferrous, ferric, aluminium and bismuth salts, and the mono- and di-hexamine complexes is described. The determination of their phenol coefficients, using *Staphylococcus aureus* at 37.5°C., showed the silver and mercuric salts to have the greatest activity. Fungistatic activity was investigated using the cup-plate technique and five organisms. The mono- and di-hexamine complexes showed the greatest activity against *Epidermophyton floccosum*, *Microsporum canis* and *Trichophyton purpureum*; the magnesium salt also had a considerable effect on the growth of these organisms. The mercuric

salt was the most effective against *Tricophyton mentagrophytes* and also had a considerable effect on *E. floccosum* and *T. purpureum*. *Candida albicans* was the most resistant of the organisms studied, only the bismuth and magnesium salts having any inhibitory effect. The silver, zinc, ferrous, ferric and lead salts had no fungistatic activity against any of the organisms.

G. R. K.

Trichothecin: an Antifungal Metabolic Product of *Trichothecium roseum*
Link. G. F. Freeman and R. I. Morrison. (*Nature*, 1948, 162, 30.) The substance responsible for the antifungal activity in cultures of *Trichothecium roseum* has been isolated in crystalline form in yields of 20 to 30 mg./l. The name "trichothecin" is suggested for the active substance; after extraction with ether or chloroform from the culture filtrate it was purified chromatographically. From light petroleum it crystallised as colourless needles m.pt. 118°, $[\alpha]_D^{18} +44^\circ$. On the basis of micro-analytical results the formula $C_{12}H_{18}O_4$ is suggested. The compound is neutral, only slightly soluble in water, contains one ketonic and one ethylenic group and the results suggest three CH_2CH groups. The antifungal activity of this compound is shown against Fungi Imperfecti, Zygomycetes and Ascomycetes. Aqueous solutions of trichothecin were stable at pH 1 to pH 10 for at least 48 hours at 20°C. At pH 12 the antifungal activity was rapidly destroyed even at room temperature.

R. E. S.

BIOCHEMICAL ANALYSIS

Cup-Plate Method in Microbiological Assay, with special reference to Riboflavine and Aneurine. A. L. Bacharach and W. F. J. Cuthbertson. (*Analyst*, 1948, 73, 334.) An assay procedure is developed which follows the principles of the cup-plate assay of penicillin. The medium is made deficient in the single substance to be assayed and the organism chosen is one that will not grow in its absence. Solutions containing the missing substance are put into the cup and after incubation, a "zone of exhibition" is shown, the diameter of which may show a relation to the concentrations of the added nutrient. Conditions are described for producing sharply defined zones of growth together with the results of investigations into the relations between inoculum density, concentration of test solution and diameter of growth zone. Details of the procedure for the determination of riboflavine and aneurine are given. The relative insensitivity and potentialities of the method are discussed.

R. E. S.

Histamine, an Improved Colorimetric Method for the Estimation of. S. M. Rosenthal and H. Tabor. (*J. Pharmacol.*, 1948, 92, 425.) Attempts to utilise the diazo reaction given by imidazole compounds for the estimation of histamine have shown it to be unsatisfactory because of lack of specificity, instability of colours and because of numerous substances in biological extracts which inhibit or interfere. To overcome these difficulties and extend the sensitivity of the test, the authors compared a large number of aromatic amines and finally selected the diazonium salt of 4-nitroaniline as the most satisfactory. The coloured azo compounds formed by 4-nitrodiazobenzene in alkaline solution are extracted with an organic solvent, which concentrates and stabilises the colour. At a suitable pH, using certain solvents, the azo compounds of most interfering substances either remain in the aqueous phase or pass into the solvent, methyl isobutyl ketone, with a yellow or amber

colour, while that of the histamine goes into the solvent with a rose colour. Ammonia reacts like histamine to give a rose colour in the solvent, but on shaking the solvent with a barbitone buffer of pH 7.7 the rose colour obtained with histamine or acetylhistamine is intensified, while that of ammonia and other interfering substances is abolished or changed to a pale yellow. A method for overcoming the effects of inhibitory substances, including those in liver extracts and urine, is described; preliminary results on various tissues and with histamine indicate a satisfactory degree of specificity for the method, which has a sensitivity of approximately 0.5 mg. S. L. W.

Penicillin, Determination of, by Alkaline Hydrolysis. Stella J. Patterson and W. B. Emery. (*Analyst*, 1948, 73, 207.) The alkaline hydrolysis method has been modified for routine assays on a large number of samples of solid penicillin having a high potency. Several indicators, singly and in combination, were tried, to avoid the use of a pH meter; cresol red, which gives a sharp colour change at the required pH even in the presence of the yellow pigment of commercial penicillin, was finally chosen. For the assay, 0.1 g. to 0.2 g. of penicillin, accurately weighed, was dissolved in 50 ml. of distilled water, previously boiled for 15 minutes to remove carbon dioxide, and cooled in a flask fitted with a soda-lime tube. 1.0 ml. of 0.1 per cent. neutral solution of cresol red in alcohol (70 per cent.) was added and $N/10$ sodium hydroxide run in slowly from a micro-burette, delivering drops of from 0.02 to 0.03 ml., until a red colour was obtained; a further 10 ml. of $N/10$ sodium hydroxide was added, the flask was stoppered with a rubber bung and the mixture was left for 3 hours at room temperature. 10 ml. of $N/10$ hydrochloric acid was then added, and the excess of acid was immediately back-titrated with $N/10$ sodium hydroxide, till the indicator changed to the original red colour. The difference between this reading, and a blank, carried out in exactly the same manner but omitting the penicillin, multiplied by 59,340 gives the total number of I.U. in the sample, and the I.U./mg. can then be calculated. The factor 59,340 applies only to salts of penicillin G; quantities of penicillin K, or of other penicillins, which are present in commercial samples, will affect the accuracy of the results. This method has been used to investigate the stability of penicillin. There are several limitations to the method; it cannot be used to assay the official ointment (500 I.U./g.) or lozenge (500 I.U.) and is applicable only for powders containing more than 900 I.U./mg. Also samples which showed no biological activity still indicated considerable potency when assayed chemically, and it is, therefore, necessary to confirm the results by biological methods from time to time. For penicillin in oil and beeswax the penicillin is separated by extraction with dry anaesthetic ether. The authors also describe an alternative procedure, using α -naphthophthalein as indicator. L. H. P.

Penicillin G in Small Broth Samples, Estimation of. J. A. Thorn and M. J. Johnson. (*Anal. Chem.*, 1948, 20, 614.) The method described is based on the fact that, on a column of Super Filtril (an acid-treated bentonite), penicillin G is more strongly adsorbed under given conditions than any other known penicillin and may be eluted as a separate fraction. The method is particularly applicable to fermentation broths and is not affected by the number of types of penicillins occurring in the sample to be analysed. The method is not intended for use on purified samples, for which the more accurate physical and chemical methods are available. A broth liquid adjusted to pH 4.6 with 50 per cent., phosphoric acid is used, diluted if necessary with 0.05M potassium monobasic phosphate so as to contain between 50 and 200 units of penicillin

per ml. The column is washed through with a phosphate buffer solution adjusted to pH 6.1, the elution of the penicillin occurring in the following sequence: first, X and dihydro F; second, F; last, G. Penicillin K appears to be largely if not entirely inactivated upon adsorption. The average recovery of adsorbed penicillin G varied from 75 to 90 per cent., depending on the adsorbent. A factor is thus needed for a particular adsorbent; this is obtained by adsorbing known amounts of penicillin G. The application of aqueous chromatography to resolution of mixtures of the other known penicillins is described as well as the effect of other acids as eluents. R. E. S.

Pregnanediol, Rapid Method for Estimation of. J. Rabinovitch. (*Nature*, 1948, 161, 605.) Pregnanediol can be detected rapidly by a method using zinc dust to protect the hormone during acid hydrolysis, and to prevent discoloration which would affect the final colour reaction. 1.5 g. of zinc dust is added to 100 ml. of urine and the mixture is heated to boiling-point, when 10 ml. of concentrated hydrochloric acid is added and the mixture boiled for 5 minutes. The flask is immersed in cold water and the zinc allowed to settle. The supernatant liquid is poured on to a sand column, and the residue of zinc is washed successively with 25 ml. of N/1 hydrochloric acid, 25 ml. of N/10 hydrochloric acid and three quantities each of 25 ml. of water; the washings are poured on to the sand column, and the column is dried by sucking hot air through it. The residual zinc is shaken with three quantities, each of 20 ml. of hot alcohol (95 per cent.), and the hot extract is decanted on to the sand column. The alcoholic extract is passed rapidly through the sand and the filtrate is evaporated to dryness. The residue is dissolved in a mixture of 5 ml. of alcohol (95 per cent.) and 20 ml. of N/10 sodium hydroxide, and allowed to stand in the cold for 1 hour. A precipitate, which contains the pregnanediol fraction, is formed, and is separated by filtration through the sintered glass, washed with water and dried. It is extracted with 10 ml. of hot alcohol, the solution may be used for the Guttmann colour reaction. In this the solution is evaporated and the residue dissolved in 5 ml. of concentrated sulphuric acid, a deep yellow or orange colour quickly develops if more than 0.5 mg. of pregnanediol was present in the original 100 ml. of urine. A negative result is shown by a colourless or pale yellow solution. The method may also be used for quantitative estimations by using larger quantities of urine and gravimetric methods. The sand-zinc method gives results of practically the same order as the quantitative method of Astwood and Jones, and is rapid and easy to carry out. L. H. P.

Tryptophane, Methionine, Cystine and Tyrosine, A Modified Method for the Microbiological Assay of. E. C. Barton-Wright and N. S. Curtis. (*Analyst*, 1948, 73, 330.) In this modified method, peptone is treated with hydrogen peroxide to destroy these 4 amino-acids and the resulting product is substituted in the basal medium for the usual series of individual amino acids. Details of the treatment of the peptone with hydrogen peroxide are given and the individual assay media are described. The organism recommended for the tryptophane assay is *Lactobacillus arabinosus* 17/5, the incubation temperature is 30°C. and the range of tryptophane to establish a standard curve is 2 to 10 µg. For the assay of L-methionine, L-cystine and L-tyrosine the organism used was *Leuconostoc mesenteroides* P.60. The range of L-methionine was 15 to 40 µg., that of cystine 5 to 35 µg., and that of tyrosine 10 to 50 µg., to establish a standard curve. The assay of DL-methionine was accomplished using *Lactobacillus fermenti* 36. Protocols of typical standard curves are given. R. E. S.

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CHEMOTHERAPY

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PHARMACY

DISPENSING

Folic Acid in Liquid Prescriptions. S. Scheindlin. (*Amer. J. Pharm.*, 1948, 120, 103.) Folic acid is unstable to oxidation, reduction, acid, alkali, dry heat, acylation esterification, methylation, benzylation, nitrous acid, bromine, hypobromite, hydroxylamine, zinc dust and acetic acid (stable to acetic acid alone) and 1 per cent. hydrogen peroxide, and it is unstable to light but not destroyed by autoclaving in the dark. Folic acid is only very slightly soluble in water, but its sodium salt is water-soluble. An elixir of folic acid containing 5 mg. of folic acid in 4 ml. of a solution containing 10 per cent. of alcohol, artificial red colour, flavouring agents and preservatives. The folic acid was present as the sodium salt and the pH was 8.2. A series of mixtures with a wide range of commonly used medicaments was then prepared and stored in clear glass bottles exposed to electric light (but not to direct sunlight) for the first week, and then transferred to a box where they were protected from light. The mixtures were examined for precipitation, colour change, liberation of gas, or change of pH after 1 day, 1 week, and 1 month. Varying concentrations of alcohol, glycerin and propylene glycol produced no change in any of the solutions after one month. At and below pH 4.1 immediate precipitation of folic acid occurred; at pH 5.1 the mixture remained clear for over 24 hours, but at the end of 1 week some precipitation had taken place. No change was observed at any other pH. Mixtures of folic acid elixir with the following drugs showed a precipitate which could not be easily suspended or which contained a potent medicament, and the author recommends that such mixtures should not be prescribed:—phenobarbitone, chloral hydrate, tinctures of hyoscyamus, stramonium, nux vomica and digitalis, and quinine dihydrochloride; in addition, sulphadiazine was found to destroy folic acid activity very rapidly. S. L. W.

GALENICAL PHARMACY

Suppository Bases, Examination of Physical Characters of. P. Malangeau. (*Ann. pharm. Franc.*, 1948, 6, 50.) The essential requirements of a suppository are: (1) that it should melt at a sufficiently low temperature to become liquid in the rectum (without being water-soluble), and (2) that it should offer sufficient mechanical resistance to enable its easy introduction. A simple method of determining the melting-point is as follows: when the melted mass is poured into the mould, a thin, polished metal rod is placed upright in the centre of the suppository cavity and maintained in that position until the suppository has set, so that when the mould is unscrewed the suppository is fixed on the end of the rod. The rod with the suppository attached is then placed in a water-bath containing 2 l. of water, the suppository being placed

CHEMOTHERAPY

Curariform Activity of Certain Chondrodendrine Derivatives. D. F. Marsh, C. K. Sleeth and E. B. Tucker. (*J. Pharmacol.*, 1948, 93, 109.) The authors compared the activity of *d*-N-methyl-chondrodendrine iodide and *d*-*o*-methyl-N-methyl-chondrodendrine iodide with *d*-tubocurarine chloride pentahydrate and *d*-*o*-methyltubocurarine iodide trihydrate in rats, rabbits, cats and man. All these compounds produce skeletal muscular paralysis and differ only in quantitative activity. *d*-N-Methyl-chondrodendrine is about one-half as active as the isomeric *d*-tubocurarine in rats and rabbits but only about one-fourth to one-eighth as active in cats and man. Although the *d*-*o*-methyl-N-methyl-chondrodendrine is about equipotent with *d*-tubocurarine, it is only one-sixth to one-eighth as active as its diastereoisomer, *d*-*o*-methyltubocurarine. S. L. W.

Curariform Activity of *iso*Chondrodendrine Derivatives. D. F. Marsh and M. H. Pelletier. (*J. Pharmacol.*, 1948, 92, 454.) A comparison with *d*-tubocurarine chloride pentahydrate and *d*-*o*-methyltubocurarine iodide trihydrate in rats, rabbits and cats, showed *d*-N-methyl-*iso*chondrodendrine to be about a twentieth, and *d*-*o*-methyl-N-*iso*chondrodendrine about a fourth as paralyzing as *d*-tubocurarine, which in turn is only about one-tenth as active as *d*-*o*-methyltubocurarine. Like the tubocurarine compounds, these *iso*-chondrodendrine derivatives have relatively little effect in intact animals other than lissive action on skeletal muscles. The authors obtained the *d*-*iso*chondrodendrine from *Pareira brava* by a modification of the method of King (*J. chem. Soc.*, 1940, 737) and prepared the derivatives as the iodide salts by the method of Dutcher (*J. Amer. chem. Soc.*, 1946, 68, 419). S. L. W.

Curarising Properties of R.P.3697. R. Wien. (*Arch. int. Pharmacodyn.*, 1948, 77, 96.) This compound, the triethyl iodide of tri(diethyl-amino-ethoxy):1:2:3 benzene, was studied as a possible substitute for *d*-tubocurarine. Its curarising properties were assayed in comparison with *d*-tubocurarine by the rabbit head-drop method, the frog rectus abdominis preparation, the cat sciatic gastrocnemius preparation, and the rat, rabbit or kitten phrenic nerve-diaphragm preparations. The results of the assays showed that by the rabbit head-drop method it was one-third as active as *d*-tubocurarine, on the frog rectus abdominis preparation it was only one-twentieth as active, and on the rat phrenic nerve-diaphragm only one-eightieth as active; on the rabbit and kitten phrenic nerve-diaphragm, however, it was one-fifth as active. The curarisation effects were easily reversed by neostigmine or eserine. Compared with similar doses of *d*-tubocurarine there was no effect on blood pressure and less effect on respiration in rabbits anaesthetised with ether and thiopentone, in chloralosed cats and in decerebrate preparations. Unlike some other synthetic curarising compounds it compared very favourably with *d*-tubocurarine for its absence of anticholinesterase properties. S. L. W.

Thio-antimonials, Organic, in Schistosomiasis. L. W. Clemence and M. T. Leffler. (*J. Amer. chem. Soc.*, 1948, 70, 2,439.) Oil-soluble substances of the general formula (RS)₃Sb have been prepared, where R may be *n*-octyl, *n*-decyl, *n*-undecyl, *n*-dodecyl, *n*-tetradecyl, *n*-hexadecyl, *n*-octadecyl, β -phenylethyl, β -(1-naphthylethyl), β -(*p*-di-isobutylphenoxy-ethoxy)-ethyl, β -cyclohexylethyl, ω -cyclohexylamyl, ω -(β -tetralyl)-butyl, ω -(β -decyl)-butyl, or β -(2-pyridyl)-ethyl. These substances are prepared by the

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reaction of mercaptan and antimony trichloride in chloroform, and show some promise in preliminary experiments on schistosomiasis. During the investigation the following substances, not previously described, were synthesised and characterised: ω -(β -tetralyl)-butyl and ω -(β -decalyl)-butyl alcohols; β -cyclohexylethyl, ω -cyclonexylamyl, ω -(β -tetralyl)-butyl and ω -(β -decalyl)-butyl isothiuronium bromides and mercaptans. G. B.

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at the same level as the bulb of a thermometer and immersed under not less than 3 cm. of water. The temperature of the water is then gradually raised, at the rate of 1° every 2 or 3 minutes, to 30°C., the water being stirred mechanically. The melting-point is taken when the mass of the suppository slides off the metal rod. For the determination of mechanical resistance, the author describes a simple apparatus, by means of which a solid cylinder of the suppository is subjected to varying degrees of vertical pressure at different temperatures. Experiments conducted with this apparatus, using cocoa butter, cocoa butter with the addition of propyleneglycol stearate, hydrogenised oil with stearates of propyleneglycol and triethyleneglycol, and hydrogenised oil with propyleneglycol stearate, showed that, whereas at ordinary temperatures the mechanical resistance of these four bases is fairly comparable, at higher temperatures cocoa butter loses its mechanical resistance much more quickly and is less likely to lend itself to the manufacture of suppositories containing a high percentage of liquid ingredients.

S. L. W.

Tablet Disintegration Testing. V. M. Filleborn. (*Amer. J. Pharm.*, 1948, 120, 233.) Tablets are immersed for a definite time in an artificial saliva bath, and enclosed in a plastic tablet container which is placed in a glass vessel containing artificial gastric juice, agitated by a pump and maintained at 37°C. Fresh artificial gastric juice is admitted by a drip-feed, and the excess allowed to flow out of the vessel. Particles of sterilised sponge may be added to simulate the presence of food. Disintegration is regarded as complete when the tablet is broken into pieces small enough to pass through the 1/16th inch holes of the plastic tablet container. Tests in which the disintegration of radio-opaque tablets, which have been swallowed whole, is observed in human subjects, show that the disintegration times obtained by the "artificial stomach" method are approximately the same as those in the human stomach. When phenobarbitone, ephedrine hydrochloride, mepacrine hydrochloride, sulphathiazole, sulphapyridine, sulphanilamide and sulphadiazine tablets are submitted to the disintegration tests of the Swiss Pharmacopœia and of the 7th Addendum to the British Pharmacopœia, 1932, the observed disintegration times are generally smaller than for the "artificial stomach" method and there appears to be no relationship between the results obtained by the three methods.

G. B.

PHARMACOLOGY AND THERAPEUTICS

Amellin Ineffective in Diabetes. H. Whittaker. (*Brit. med. J.*, 1948, 1, 546.) A mixture of amellin (an extract of *Scoparia dulcis*), calcium gluconate and lactose was given to 2 patients with diabetes in doses of 5 gr. (0.32g), by mouth, thrice daily for 3 months. One patient also received insulin and the progress of his disease was unaltered by the administration of amellin. The patient receiving amellin without insulin became progressively worse and heavy glycosuria and hyperglycæmia were constantly present. After 3 months' treatment with amellin, traces of ketone bodies were found, and the blood-sugar was 348 mg./100 ml. After doses of 16 units of protamine-zinc-insulin daily, the urine became sugar-free and blood sugar 4 hours after breakfast was 206 mg./100 ml.

G. R. B.

Amidone (Methadon), Clinical Evaluation of. Elizabeth B. Trioxil. (*J. Amer. med. Ass.*, 1948, 137, 920.) Amidone (physeptone) was administered by mouth, as capsules, tablets, or elixir, or by hypodermic or intravenous

injection. Onset of action occurred in 2 minutes after intravenous injection, 15 to 20 minutes after hypodermic injection or administration as elixir, and 30 minutes after administration as capsules or tablets; the average duration of action by all routes was 3 to 4 hours, but sometimes the effect lasted for 8 to 12 hours. Usually the hypodermic route was used, but the elixir was found to be equally efficacious and more suitable for prolonged use. When tested on a group of 400 patients showing all degrees of pain from a variety of clinical conditions, amidone gave complete and adequate relief to 81 per cent. in doses varying from 2.5 to 20 mg. Side-effects occurred in 13 per cent. of the patients and included nausea and vomiting, sedation (generally a slight drowsiness) and dizziness. When compared with morphine and pethidine on a group of 90 patients, the analgesic effect of 10 mg. of amidone was found to be equivalent to that of 15 mg. of morphine or 150 mg. of pethidine. No evidence of addiction was encountered in three patients who were treated with the drug for one year. Morphine, pethidine, "pantopon" and dihydromorphine addicts experienced no withdrawal symptoms when the narcotic was replaced with amidone, or after the end of treatment. No contraindications were met with, but the routine use of elixir in patients with dysmenorrhœa was not encouraged because of the high incidence of nausea and vomiting. For the relief of obstetric pain, amidone is inferior to pethidine.

G. R. K.

Arsenicals, an Improved Method for Assay of Toxicity. W. L. M. Perry (*Nature*, 1948, 161, 975.) In a quantal response assay, each animal can contribute only a positive or a negative reading (in this case, death or survival), and in the event of death there is no indication whether the dose given was the exact minimum individual lethal dose, or whether it was considerably in excess. Thus, the method is wasteful of information, and only when a continuous variate such as survival time cannot be used is recourse to quantal response methods necessary. In the case of the arsenicals there seems to be no such difficulty. Using the survival time as the continuous variate it has been found possible to perform an assay with increased speed, accuracy, and economy in animals; a definite numerical estimate of the toxicity of the drug for that particular animal is obtained, and provided a linear dose-response relationship can be employed it is to be expected that more information will be gained per animal used. A series of experiments with neoarsphenamine, so designed that the methods of quantal responses and measurement of survival times could be compared, was carried out. The dose range in the latter case was chosen to ensure that all the animals treated should die, and that the longest period of survival should not exceed 10 hours. A linear relationship between the dose of drug and mean survival time was established by using logarithmic transformations. Statistical analysis of the results shows the graded response method to be accurate and unbiased. The limits of error for the graded response assay were shown to be about half as wide as those for the quantal response assay, and the accuracy of the estimation of potency about 4 times as great.

S. L. W.

Atropine Poisoning, Acute. R. B. Welbourn and J. D. Buxton. (*Lancet*, 1948, 255, 211.) A report of 9 cases of acute atropine poisoning, arising from a dispensing error, 1/6 grain of atropine sulphate being given by subcutaneous injection, instead of the 1/100 grain prescribed pre-operatively. All the patients were young men with septic conditions requiring minor operations, and the poisoning was not suspected until after operation. Only 4 of the patients showed toxic effects, namely, acute delirium and blurred vision,

and all recovered completely. In these 4 cases difficulty was experienced in anaesthetising with soluble thiopentone and nitrous oxide and oxygen. Of the remaining 5 cases, who showed no toxic effects and in whom no difficulty was experienced in producing anaesthesia, 3 were anaesthetised with trichloroethylene which has a more powerful and lasting depressive action on the central nervous system than nitrous oxide and oxygen or soluble thiopentone. S. L. W.

Aureomycin; Experimental and Clinical Investigations. M. S. Bryer, E. B. Schoenbach, C. A. Chandler, E. A. Bliss and P. H. Long. (*J. Amer. med. Ass.*, 1948, 38, 117.) Aureomycin is supplied as the hydrochloride of an antibiotic from a strain of *Streptomyces aureofaciens*. It consists of yellow crystals, soluble in water giving a solution of pH about 4.5, and slightly less soluble in isotonic sodium chloride solution. Alkaline solutions are unstable. *In vitro*, 0.1 to 5.0 $\mu\text{g./ml.}$ inhibits the growth of various Gram positive and Gram negative bacteria, but *Pseudomonas aeruginosa* and strains of *Proteus* are unaffected by 20 $\mu\text{g./ml.}$ Fifty times the concentration is required when 50 per cent. of human serum is present. Mice treated orally with 50 mg./kg. of body weight are protected against β -haemolytic streptococci (C203), but not against *Klebsiella pneumoniae* A. and *Diplococcus pneumoniae* I (S.V.I.). In human patients, coli-aerogenes and *Streptococcus faecalis* infections of the urinary tract are sterilised by 10 to 60 mg./kg. of body weight per day, orally. Favourable initial responses are obtained in patients with Rocky Mountain spotted fever, and with brucellosis, using 3 mg./kg. per day, intramuscularly. G. B.

Cycloheptenylethylbarbituric Acid, Toxicology and Pharmacology of. W. A. Halbeisen, C. M. Gruber, Jr., and C. M. Gruber. (*J. Pharmacol.*, 1948, 93, 101.) The intraperitoneal LD50 of cycloheptenylethylbarbituric acid (medomin) is 284 mg./kg. for mice and 220 mg./kg. for rats. When injected intravenously it is 119 mg./kg. for rabbits and 105 mg./kg. for dogs; both rabbits and dogs appeared to develop a tolerance for the drug. Large doses rapidly given intravenously produce a sudden fall in arterial pressure, the extent being directly proportional to the amount given and the speed of administration. An increased heart rate occurs during the fall and persists for some minutes after blood pressure has returned to the control level. Large doses given rapidly intravenously cause marked slowing of, and may permanently stop, respiration in expiration; the respiratory mechanism fails before the heart. When the fall in blood pressure is not extensive there appears to be dilatation of the vessels of the spleen, intestine, kidney and limb; when it is sudden and extensive, a decrease in the volume of these organs is observed, which the authors believe to be passive in character. There is dilatation of the vessels of the skin. The drug appears to have a less depressant effect on the cardiac vagus nerves than other intermediate-acting barbiturates such as amytal sodium. Like other intermediate-acting barbiturates it is destroyed in the body and is excreted as the parent substance only when excessively large doses are given. S. L. W.

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exposed and cannulated, the cannula consisting of a blunted 22-gauge hypodermic needle, and the test solution injected from a 10-ml. micro-burette calibrated to 0.1 ml. Very light ether anaesthesia was maintained throughout the assay. The solution to be assayed was diluted so that the estimated fatal dose per kg. was diluted to 15 ml. with 0.9 per cent. sodium chloride solution. When the average death time fell outside 60 to 90 minutes a new dilution was prepared. As the pigeon is *slightly more resistant to digitalis* than the cat, more tincture per 100 ml. is required; an additional 1 ml./100 ml. of tincture usually suffices. The diluted tincture was injected at the rate of 0.1 ml./100 g. of pigeon at 5-minute intervals until cardiac arrest supervened; the end point is very sharp. At least 6 pigeons were used for every preparation to be assayed. The U.S.P. requirement of a standard error of ± 5.7 per cent. was adhered to, and 6 to 8 pigeons were usually sufficient to come within this figure. From a comparison of the results obtained with 30 preparations of digitalis it was found that the potency estimates obtained by this method varied in no case by more than 13.6 per cent. from those obtained by the present U.S.P. method; the average deviation between the two methods was not significantly different from zero. The coefficient of variation of the lethal dose of digitalis for the pigeon was 10.4 per cent., compared with 12.9 per cent. for cats. Preliminary investigations show that not only are pigeons more consistent in a given assay than cats but that various batches of pigeons seem to vary less from each other than various batches of cats.

S. L. W.

Dimercaprol (B.A.L.) Treatment of Gold Dermatitis. N. R. W. S i m p s o n. (*Brit. med. J.*, 1948, 1, 545.) A 5 per cent. preparation in arachis oil with 10 per cent. of benzyl benzoate was administered by deep intramuscular injection in the treatment of two cases of gold dermatitis. 2 ml. was given 4 times on the first day, 2 ml. thrice daily for 3 days, 2 ml. once daily for 9 days and subsequently, 2 ml. every alternate day. In one case, redness, heat, pain and induration occurred at the site of injection. After treatment was commenced, a lapse of 6 days occurred before improvement in the dermatitis was noted.

G. R. B.

Fluorescein as an Indicator of Antihistamine Activity. S. C. B u k a n t z and G. J. Daurmin. (*Science*, 1948, 107, 224.) Fluorescein was used as a tracer substance to investigate the changes in capillary permeability due to antihistamine activity in the skin. The first experiment, determining the fluorescence at skin sites of a dog, showed that the antihistamines NH188 (neohetramine) and benadryl were of approximately equal activity in preventing fluorescence. In a second experiment, fixed concentrations of histamine in varying concentration of the antihistamine drugs NH188 (neohetramine) or benadryl were injected intradermally into each of 5 human subjects and 3 ml. of a 5 per cent. solution of fluorescein was injected intravenously soon afterwards. It was found that there was an inverse relationship between the concentration of the antihistamine drug and intensity of fluorescence; also at dimly fluorescent sites the initial fluorescence took longer to develop and was of shorter duration than at highly fluorescent sites. To determine the effects of histamine and of antihistamines on the rate of absorption of fluorescein injected intradermally, fluorescein (1 in 50,000 of saline solution), fluorescein + histamine (1 in 10,000), and fluorescein + histamine (1 in 10,000) + benadryl (1 in 2,000) were injected into three sites on the forearms of 3 normal and 1 allergic human subject. The fluorescein sites remained visible under ultra-violet light for 30 to 45 minutes in the normal cases, while the fluorescein + histamine sites no longer

and all recovered completely. In these 4 cases difficulty was experienced in anaesthetising with soluble thiopentone and nitrous oxide and oxygen. Of the remaining 5 cases, who showed no toxic effects and in whom no difficulty was experienced in producing anaesthesia, 3 were anaesthetised with trichloroethylene which has a more powerful and lasting depressive action on the central nervous system than nitrous oxide and oxygen or soluble thiopentone. S. L. W.

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fluoresced after 4 to 10 minutes; the fluorescein + histamine + benadryl site fluoresced as long as the fluorescein sites. In the allergic subject the fluorescence of the fluorescein and the fluorescein + histamine sites disappeared within 4 minutes, but that of the fluorescein + histamine + benadryl site remained for 25 minutes. This indicated that normally fluorescein is rapidly absorbed under the action of histamine but that benadryl antagonises this action; in allergic subjects a histamine-like substance released locally causes rapid absorption of fluorescein from the skin and this also is neutralised by the presence of the antihistamine drug. The time for appearance, intensity and duration of fluorescence at histamine-injected sites may thus be quantitatively modified by the local presence of antihistamine substances.

L. H. P.

Gonadotrophin. Crystalline Human Gonadotrophin and its Biological Action. L. Claesson, B. Hogberg, T. Rosenberg and A. Westman. (*Acid endocrinol.*, 1948, 1, 1.) A crystalline and electrophoretically homogeneous form of chorionic gonadotrophin was isolated from the urine of pregnant women by a method which is fully described. It possesses a constant biological activity of 6,000 to 8,000 I.U./mg. It shows a marked stimulatory action on the growth and maturation of the follicles and on the formation of corpus luteum in intact mice, rats and rabbits, but fails to do so in hypophysectomised rats; in this latter group the crystalline hormone produces only an extensive development of the ovarian interstitial gland. Administered intravenously, it is well tolerated by patients in daily doses as high as 12,000 I.U. injected on 3 consecutive days, causing increased follicular growth in the human ovary and a forced production of oestrogenic hormones. In amenorrhoea due to pituitary hypofunction, large doses intravenously may induce bleedings from the progestational endometrium. Combined with small doses of serum gonadotrophin from pregnant mares it produces intensive development of the follicles. The granulosa and theca cells show no sign of degeneration. Follicular rupture and corpus luteum formation takes place in contrast to the effects induced by the action of crystalline chorionic gonadotrophin administered alone.

S. L. W.

Intestinal Carminatives. Method for Assessing Value. S. A. Istead and J. Fleming Patterson. (*Lancet*, 1948, 254, 437.) A simple method for assessing the value of carminatives for expediting the passage of flatus from the bowel is described. A rubber catheter was passed about 2 inches beyond the anal sphincter. The free end was connected with a glass adapter to a piece of rubber tubing, and the tubing attached to a 500 ml. glass measuring cylinder, inverted in water to act as a gas jar. As bubbles of gas displace the water column, the volume of gas is recorded. The oral administration of a carminative mixture, hot turpentine stupes to the abdomen, radiant heat and the injection of carbachol were found by this test to be ineffective. Pituitary extract was found to be the most valuable; physostigmine and prostigmine were only occasionally effective in increasing the output of flatus.

G. R. B.

Myanesin, Relaxant in Children. W. H. Armstrong Davison. (*Brit. med. J.*, 1948, 1, 544.) A dose of myanesin (α : β -dihydroxy- γ -(2-methylphenoxy)-propane) of the order of 2 ml. per stone (6.36 kg.) of body weight was given to 44 children between the ages of 24 days and 4½ years to obtain relaxation for abdominal surgery. Maintenance anaesthesia was with open ether, nitrous oxide, or ethyl chloride. Relaxation after the dose

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of myanesis occurred rapidly and was maintained for 10 to 25 minutes. The injection was made into the intravenous drip, if one was set up, or into the longitudinal sinus at the posterior angle of the anterior fontanelle.

G. R. B.

Neohetramine, A New Antihistamine Drug, Pharmacological Characteristics of. N. B. Dreyer and D. Harwood (*Proc. Soc. exp. Biol.*, N.Y., 1947, 66, 515.) The amount of neohetramine required to abolish the contractions of guinea-pig ileum and uterus, and cat uterus produced by a concentration of 0.03 to 0.3 μ g. of histamine base in oxygenated Ringer solution was determined. The ratio of the amount of neohetramine to histamine was then calculated. This was 2.7:1 for guinea-pig ileum, 1.4:1 for guinea-pig uterus and 0.7:1 for cat uterus. In dogs and cats the fall in blood pressure caused by 1 to 2 μ g. of histamine was offset by 2.5 mg./kg. of neohetramine. However, the effect of larger doses of histamine was not neutralised. Neohetramine, like pyribenzamine, did not affect the inhibition of rat uterus by histamine, but histamine-induced vasoconstriction in a perfused rabbit ear was counteracted by an equivalent concentration of the drug. Doses of 1 to 5 mg./kg. of neohetramine given to atropinised cats and dogs caused an immediate drop in blood pressure without a change in the heart-rate. The animals recovered in a few minutes. Neohetramine showed little or no effect on sympathetic nerve stimulation. On the parasympathetic nerve system, neohetramine exerted some atropine-like action on the chorda tympani, but even large doses failed to abolish chorda secretion. Neohetramine did not lessen the effect of the vagus on the intestine, and in some cases seemed to potentiate it. The total and free acidities of gastric juice obtained by rhythmic stimulation of the left vagus were unaltered by doses of neohetramine up to 5 mg./kg.

A. D. O.

Podophyllin, Effect of, on Transplanted Mouse Tumours. M. Belkin, (*J. Pharmacol.*, 1948, 93, 18.) Podophyllin dispersed in sesame oil was given subcutaneously in doses of 20 mg./kg. to 12 mice carrying 15-day-old implants of sarcoma 180, a similar group of mice given injections of sesame oil alone serving as controls. Injections were made every 3 or 4 days for 2 weeks on the side opposite the tumour. The controls grew typically, but the tumours in mice receiving podophyllin exhibited a prompt decrease in growth rate; at the end of 2 weeks the average volume of the treated tumours was approximately one-seventh that of the controls. In another experiment similarly conducted the effect of podophyllin was tested on a mammary adenocarcinoma. In this case the terminal volumes of the treated mammary tumours was approximately two-thirds that of the controls. For both kinds of tumours used in these experiments the most prominent and consistent histological finding following podophyllin administration was extensive necrosis. Characteristic nuclear alterations were found in both types of tumour. The podophyllin produced varying degrees of malaise, and diarrhoea. Resistance to repeated administration does not develop, judged by the appearance of the tumours after several injections of the drug. S. L. W.

Procaine Penicillin G. W. E. Herrell, D. R. Nichols and F. R. Heilman. (*Proc. Mayo Clin.*, 1948, 22, 567.) In the search for methods of prolonging the effective concentration of penicillin in the blood the authors examined the properties of a procaine salt of penicillin G (duracillin). Procaine penicillin G is a crystalline, non-pyrogenic substance prepared by combining one molecule of procaine base with one molecule of penicillin. The resulting compound contains 41.5 per cent. of procaine base and has

a potency of 940 units/mg. It is employed in the form of a suspension in sesame oil, each ml. of the suspension containing 300,000 units of penicillin and 125 mg. of procaine. After preliminary animal experiments to ascertain the non-toxicity of the preparation, intramuscular injections of 1 ml. were given to 10 patients. Determination of the concentration of penicillin in the blood, either by the Fleming slide-cell technique or by the Kolmer serial dilution method, disclosed an effective therapeutic concentration for at least 24 hours after the administration of the injection. The injection appears to be safe and non-toxic; no local irritation, soreness or pain followed. Therapeutic results are the same as would be expected from any other form of penicillin therapy. It is important not to massage the site of injection.

S. L. W.

Sulphetrone in Tuberculosis. M. G. Clay and A. C. Clay. (*Lancet*, 1948, 255, 180.) Of 44 cases of tuberculosis treated with sulphetrone improvement was noted in 22, 5 were unchanged, 6 became worse, and 11 died. Of those improved, 9 improved considerably, 7 moderately, and 6 slightly. Improvement was not dramatic, and at best sulphetrone can only be regarded as an adjuvant and not in any way as a specific for tuberculosis. An attempt was made to keep the blood-sulphetrone level at 7.5 to 10 mg./100 ml. At first sulphetrone was given as long as the patient tolerated it, but later it was given in courses of 14 to 15 weeks, with a rest period of 6 weeks between courses. Parenteral sulphetrone was found to possess no advantages over sulphetrone orally. The hypochromic and nutritional anæmias were corrected by administration of ferrous sulphate 3 to 6 gr. twice daily, and yeast, preferably autolysed or boiled, 2 dr. twice daily. Changes in the alkali reserve were compensated by giving 30 gr. of sodium bicarbonate 3 or 4 times a day. Most patients developed cyanosis, but this was not an indication for stopping treatment. In most patients the treatment caused so little upset that they could continue taking it after they got up. Sulphetrone should be used only if there are facilities for estimating blood-sulphetrone levels and for carrying out blood counts.

S. L. W.

Sulphetrone, Treatment of Experimental Tuberculosis with. G. Brownlee and C. R. Kennedy. (*Brit. J. Pharmacol.*, 1948, 3, 29.) In an experiment in which two groups of 20 guinea-pigs were infected with a heavy inoculum of a virulent bovine strain of tubercle bacilli, the survival time of the group treated with sulphetrone (0.6 g. daily in the diet) was prolonged, being 77 days compared with 45 days in the untreated group. In a second experiment in which the infection was a heavy inoculum of a human virulent strain, the treated group of 24 animals survived considerably longer than the untreated group of 21 animals. Throughout the entire drug-treated group macroscopic evidence at necropsy showed very much less tuberculosis than in the untreated group. This was confirmed by histological examination, and by the observation that acid-fast organisms were very much less in number than in the untreated group. The most significant histological evidence was the repeated finding of healed tuberculous lesions, often calcified, in the spleen, liver, lungs and lymph nodes. With both the bovine and the human strains the results suggest that sulphetrone exerts a retarding effect on the progressive nature of established experimental tuberculosis in guinea pigs, though it is evident that it is incapable of eliminating the causative organism.

S. L. W.

bis-Trimethyl Ammonium Compounds, Pharmacology of. G. E. Glock. G. A. Mosey and J. W. Trevan (*Nature*, 1948, 162, 113.) The authors confirm the findings of previous workers on the curare-like action of a series

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of bis-quaternary ammonium polymethylenes. Thus, they find that the C_2 compound curarises but is relatively inactive compared with *d*-tubocurarine chloride on the rat diaphragm, and its action was completely reversed by neostigmine; the C_3 compound had no action on the rat diaphragm. The C_3 compound had marked cholinergic action, together with relaxation of decerebrate activity at a dose of 1 mg./kg.; it produces a response of the rabbit's ileum similar to that of acetylcholine; it has slight activity as an anticholinesterase, but has no action on pseudo-cholinesterase. Its cholinergic activity, and its low curarising activity, render it unsuitable as a clinical substitute for *d*-tubocurarine. The C_5 compound has about the same anticholinesterase activity as the C_3 . The overlapping of "muscarine," "nicotine" and anticholinesterase activities is a very striking phenomenon which constantly occurs in complex quaternary ammonium compounds. If the chain includes phenyl groups the effect of increasing the distance between the N^+ atoms is not to increase the curarising activity but to develop anticholinesterase activity. With regard to the species variation, results with the two closely related compounds, *d*-tubocurarine and its dimethyl ether, show that not only does the ratio of the potency vary between different species, but also, especially in the rabbit, the discrepancy may be larger in the intact animal.

S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

Iodonium Compounds, Antibacterial Activity of. L. Gershenfeld and B. Witlin. (*Amer. J. Pharm.*, 1948, 120, 158.) In iodonium compounds iodine is present as an integral part of the positive ions. They are strong bases and form stable salts. They are decomposed by heat. Hitherto, investigations of these compounds have been primarily for the preparation and synthesis from the standpoint of valency studies, and they have only recently been studied to determine whether they exert insecticidal or bactericidal effects. The authors conducted antibacterial efficiency tests on the following:—diphenyliodonium chloride, *bis-p*-chlorophenyliodonium sulphate, *bis-p*-bromophenyliodonium iodide, *bis-p*-chlorophenyliodonium iodide, *bis-p*-iodophenyliodonium iodide and diphenyliodonium iodide. These compounds, in powder form, showed bacteriostatic activity when tested by the F.D.A. agar plate technique, but only *bis-p*-chlorophenyliodonium sulphate in saturated aqueous solution showed bactericidal efficiency against *Staphylococcus aureus* at 37°C. within 1 minute. The addition of sodium thiosulphate did not affect the bacteriostatic or bactericidal efficiencies. Saturated solutions of the compounds in alcohol (95 per cent.) showed greater bactericidal efficiency than alcohol itself against *Staphylococcus aureus*, and saturated solutions in a solvent consisting of acetone 10 per cent. by volume in alcohol (95 per cent.) were also effective against this organism. A saturated solution of *bis-p*-chlorophenyliodonium sulphate in acetone-alcohol solvent showed bactericidal efficiency in 1 minute against *Eberthella typhosa*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus vulgaris*, and was capable of killing *Bacillus subtilis* (24-hour culture) and *B. subtilis* spores (4-day old culture) within 4 hours at 37°C.

S. L. W.

Iodonium Compounds, Bacteriostatic Efficiency of. L. Gershenfeld and B. Witlin. (*Amer. J. Pharm.*, 1948, 120, 170). Bacteriostatic efficiency tests were performed on the following iodonium compounds in aqueous solution:—diphenyliodonium chloride, *bis-p*-chlorophenyliodonium sulphate, *bis-p*-bromophenyliodonium iodide, *bis-p*-chlorophenyliodonium iodide and

diphenyliodonium iodide. The test organisms used were *Staphylococcus aureus*, *Serratia marcescens*, *Eberthella typhosa*, *Pseudomonas aeruginosa*, *Escherischia coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus megatherium* and *Streptococcus haemolyticus*. The minimum bacteriostatic concentration of the compounds varied from 0.001 mg./ml. for *bis-p*-chlorophenyliodonium iodide (*Staph. aureus*) to 0.8 mg./ml. for *bis-p*-chlorophenyliodonium sulphate (*Proteus vulgaris*). In the over-all picture, considering all test organisms, diphenyliodonium chloride appeared the most generally effective. It was bacteriostatic to all organisms tested, and it showed bacteriostatic efficiency at lower concentrations than any of the other compounds tested in the case of 6 of the 11 organisms for which a reasonable comparison was possible. *bis-p*-Bromophenyliodonium iodide appeared second in general effectiveness, being ineffective only against *Eberth. typhosa* and *E. coli*. It was generally effective at the relatively low concentration of 0.009 mg./ml. From a comparison of the results of diphenyliodonium chloride and iodide with those of *bis-p*-chlorophenyliodonium sulphate and iodide, it would appear that it is the anion which has the effect on the activity. Intraperitoneal injections in mice of diphenyliodonium chloride and *bis-p*-chlorophenyliodonium sulphate caused increased excitability, increased respiration, and paralysis of the hind legs. The lethal dose for both of the compounds was 20 mg./kg. of bodyweight.

S. L. W.

Quaternary Ammonium Disinfectants; a Semi-micro Method for Testing. E. G. Klarmann and E. S. Wright. (*Amer. J. Pharm.*, 1948, 120, 146.) Two factors interfere with the use of the "phenol coefficient" method in the evaluation of quaternary ammonium compounds, namely, (1) the creation of a condition in the "medication" mixture (of diluted cationic disinfectant plus bacteria), which prevents the transfer of a truly representative bacterial sample to the subculture, and (2) the failure to take due account of, and to suppress, the characteristic and marked bacteriostatic action of the cationic compounds in the transfer tube. The authors have developed a semi-micro method to overcome these factors, using Bacto-Oxgall to suppress bacteriostasis. Depending upon whether *Eberthella typhosa* or *Staphylococcus aureus* is to serve as the test organism, 1 or 5 per cent. respectively of Bacto-Oxgall is used. The composition and preparation for use with *E. typhosa* is as follows:—10 g. of Armour's peptone, 5 g. of Armour's beef extract, 10 g. of Bacto-Oxgall in 1000 ml. of distilled water; boil, and adjust to pH 7.4, and autoclave for 30 minutes. Add 5 g. of "Super-Cel" (10 g. with 5 per cent. of Bacto-Oxgall), and filter while hot. Add 5 g. of dextrose per litre, transfer to tubes each containing 20 ml., and autoclave for 30 minutes at 15 lb. pressure. The details of the semi-micro technique are as follows:—pipette 0.05 ml. of a 24-hour F.D.A. broth culture of the test organism on to the bottom of sterile 25 x 150 mm. test-tubes, taking care that the pipette does not touch the walls of the test-tube. Place the tubes in a water-bath at 20°C; add 0.5 ml. of diluted disinfectant, which has also been kept in a water-bath at 20°C, to each tube and mix thoroughly with the culture; 10 minutes later pour 20 ml. of Bacto-Oxgall broth into the tube, using aseptic precautions; incubate all tubes for 48 hours at 37°C. The results obtained by this method suggest that the quaternary ammonium compounds are not entitled to the phenol coefficient figures obtained with the original F.D.A. method; conversely, the latter method does not appear to be directly applicable to the testing of these compounds.

S. L. W.

PHARMACOPŒIAS AND FORMULARIES

THE PHARMACOGNOSY OF THE BRITISH PHARMACOPŒIA 1948

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Member of the Swiss Pharmacopœia Commission

READING the B.P. 1948, the foreign observer is struck by the up-to-date selection of the contents, so that excellent monographs for the newest synthetics and pure active principles of vegetable and animal drugs, such as penicillin and heparin, are included. On the other hand, certain vegetable drugs, of which the importance and the consumption is still quite considerable, such as cinchona, chamomile, peppermint and linseed, have been dropped. I am of the opinion that a Pharmacopœia should standardise such drugs officially, even though, as in Great Britain, semi-official standards are given by a book such as the Pharmaceutical Codex. Most of the official titles are well chosen from the botanical point of view, but for a few monographs I would suggest the replacement of the designations by the true botanical names, e.g. *Aurantii Pericarpium* or *Flavedo* would be more accurate than *Aurantii Cortex*. Further, the same designation should be used for botanically equivalent drugs, e.g. for the leaves and flowering tops of *Belladonna* and *Hyoscyamus*, which should both be called *Herba*. The definitions of the drugs are generally very clear. In certain cases, however, similar drugs differing somewhat in their properties and in their behaviour during the preparation of extracts and tinctures, are summarised in the same monograph, e.g. on *Aloe*, where both the hepatic and the vitreous types are admitted, on *Benzoin* and on *Amylum*. In the latter case, e.g. rice starch has an inner surface 10 times greater than potato starch, a fact which without doubt will influence the adsorbing effect. Insufficient attention is paid to the standardisation of conditions of harvesting and of conservation of drugs. These two operations affect the qualitative and the quantitative composition of the complex active principles in such a way that the variation will not be detected by the official assay. There are great difficulties in controlling such standardisation, but I believe that the uniformity of the drugs would be improved if directions for harvesting and conservation were included. In the few cases for which special conditions are stated, as for *Digitalis* and *Colchicum*, the requirements are on the correct scientific basis.

The standards for minimal content of active principles are in general rather low. This is for instance the case with *Carum*, *Cinnamomum*, *Colchicum*, *Filix Mas* and especially with *Coriandum* and *Fœniculum*, two drugs for which a content $\frac{1}{2}$ to $2\frac{1}{2}$ times higher could easily be prescribed. One of the main duties of a Pharmacopœia is to require high standards for drugs. Only in this way can the suppliers be forced to produce drugs of high quality. For *Digitalis Folium* no minimal standard of biological activity is prescribed. If *Digitalis Præparata* and the tincture are required to have a certain potency, it seems to be necessary to ensure at least the same, or better a somewhat higher, potency for the initial crude drug.

The macroscopical and microscopical descriptions are very good and are given in a very detailed manner. In a few instances I would have preferred, however, that more stress had been laid upon the characteristics which are essential for differential diagnosis. For example, there is an excellent detailed

description of the anatomy of the costæ in Caraway fruit but no mention made of the typical small vittæ to be found on the outer side of the vascular bundle of each costa. Although the B.P. indicates that the parquetry layer is not present in Caraway this layer actually is well developed in this fruit.

The macroscopical and microscopical sizes are worked out very carefully. By putting in "about" or "mostly" in front of the indicated sizes an important fact has been taken into account that the dimensions of organs or cells depend on the conditions of growth and other factors and therefore may vary and exceed the permitted figures. The width of the parquetry layer in cells of Umbelliferous fruits is very useful in the differentiation of these drugs and should be stated in a future revision.

The quantitative microscopical determination of foreign organic matter by the lycopodium method of Wallis is a very useful innovation; in a further edition the characteristic elements for the drug in each case should be indicated.

The methods of assay of active principles work very well; they are accurate and well planned. If I have any criticism to make it is that the British method of extraction and purification of the alkaloid takes more time than e.g. the method of the Swiss Pharmacopœia and this without improving in any high degree the accuracy of the assay. It would be advisable to assay in *Ipecacuanha* not only the total alkaloids but also the relation of emetine and cephaeline, especially as both the Rio and the Cartagena drugs are admitted, the latter containing a much higher percentage of the rather undesirable cephaeline.

All drugs containing essential oils are assayed, and it is especially valuable that the diminution of content of essential oil produced by grinding is recognised by giving different figures for the oil-content of both the whole and the ground drug.

The treatment of ash values is rather inconsistent. For several drugs (*Ergota*, *Hamamelis*, *Podophyllum*, etc.) no figure is given. In other monographs there are only figures for the total ash, in a further group of drugs there are figures for acid-insoluble ash only, and in a last group the determination of both the total ash and the acid-insoluble ash. To such much importance has been given to the acid-insoluble ash in certain pharmacopœias, especially when it is considered that this value corresponds with the external mineral impurities. My own experience is that the acid-solubility of external mineral impurities is much higher in drugs grown on calcareous soils than in drugs grown on siliceous soils.

In conclusion, I am glad to have the opportunity of saying that Pharmacognosy has been dealt with in the British Pharmacopœia 1948 in a careful and highly critical manner. The small improvements which I have suggested are given for two reasons, first, the somewhat different continental tradition in pharmacognosy and, secondly, the desire and the hope of giving some modest help to our British friends.

BOOK REVIEWS

AMERICAN PHARMACY. Edited by R. A. Lyman. Vol. 1, 2nd edition, 1948. Pp. 552, Figs. 200. Vol. 2, 1947. Pp. 379. Figs. 111. J. B. Lippincott Co., Philadelphia and London.

This work has been produced in the main by about a score of professors of pharmacy, assisted by specialists on medical, veterinary, zoological and commercial aspects. Six advisory editors, a technical editor and an editor-in-chief have undertaken the final task of production. From this it will be seen that the two volumes constitute a serious contribution to pharmacy in general. To a non-American reader it is not so clear why the subject should be designated American pharmacy. Certainly the references to original English work are somewhat scanty and it would seem that the various authors were not able to consult English pharmaceutical literature as freely as that of the U.S.A. As an illustration, the chapter on the extraction of drugs, followed as are most of the chapters by references to original papers, gives about 20 references to historically interesting work up to 1870, but only one reference to English work, i.e. the continuous extraction apparatus of Self and Corfield (1930), now official in the B.P. The many papers on extraction published in the *Quarterly Journal of Pharmacy and Pharmacology* during the last 20 years are not referred to. Volume 1 is divided into three main parts. Part I deals with Fundamental Principles and Processes. Among other subjects its 10 sections, starting with metrology, deal with the following: heat and refrigeration, purification and clarification, solution, colloids, emulsions and suspensions, extraction, bacteriological technique, preservation and packaging. Part 2 describes the various galenical preparations of the U.S.P. and of the N.F. These are classified in accordance with their chief characters, thus the mucilages, creams, glycerogelatins, glycerites and collodions are brought together. Emulsions form an important section and are treated very thoroughly with much valuable information on the newer emulgents. Part 3 of this volume is devoted to biologicals and describes the vitamins, hormones and endocrine glands, with a short chapter on other biological products, such as the antibiotics, penicillin and streptomycin, with a table of the lesser known substances. Volume 2 is divided into three main parts. (1) Advanced Pharmacy, dealing with such subjects as flavours, colouring agents, deodorants, solvents, parenteral preparations, and tablets. (2) Medical, Surgical and Dental supplies. (3) Animal Health Pharmacy. As is usual in recent American publications the volumes are exceedingly well, even extravagantly, produced. This work expresses a recognition of "the destinies of America as a teacher, administrator and adviser to the professional pharmacist" and thus represents a benign challenge to us all.

H. FINNEMORE.

BOOKS RECEIVED

BACTERIAL AND VIRUS DISEASES by H. J. Parish. Pp. 159 and Index, E. & S. Livingstone, Ltd., Edinburgh, 1948, 7s. 6d.

THE U.F.A.W. HANDBOOK ON THE CARE OF LABORATORY ANIMALS edited by A. N. Worden. Pp. XVI + 368, Balliere, Tindall and Cox, London, 1947, 31s. 6d.

LETTERS TO THE EDITOR

Colour and Fluorescence Reactions for Steroid and Synthetic Hormones.

FOR some time it has been known that warming with concentrated sulphuric acid caused condensation of several natural oestrogens with the production of coloured solutions with varying fluorescence effects. Boscott¹ has recently developed the phosphoric acid reaction of Finkelstein, Hestrin, and Koch² for the detection and estimation of steroid and synthetic oestrogens. The need for a reaction to distinguish between tablets containing small amounts of the various steroid and synthetic hormones in general has led us to investigate the possibility of extending Boscott's technique for this purpose, since it is known that the presence of small amounts of tablet disintegrants and lubricants interferes with certain colour reactions for these substances (cf. Cocking³). The basic technique described by Boscott was followed after preliminary extractions (when necessary) of the crushed tablets with ether and evaporation of the solvent. It is not certain in cases where extraction had to be used what accompanying tablet constituent was interfering with the fluorescence reaction; starch interfered with the dienœstrol reaction but in other cases merely caused a slight alteration of the fluorescence colour. The results given by a number of steroid hormones not previously examined are also recorded.

STEROID HORMONES.

The crystalline hormone was dissolved in 0.2 ml. of glacial acetic acid, mixed with about 2 ml. of 88 per cent. phosphoric acid and allowed to stand for 1 hour, the colour and fluorescence (under filtered ultra-violet light) being observed at intervals. After 1 hour the solution was diluted with about 3 ml. of glacial acetic acid and the colour and fluorescence again noted. In addition, approximate fluorescence intensities are reported relative to œstrone as standard.

Desoxycorticosterone acetate. 1 mg. gave a violet fluorescence, weaker than œstrone after 1 hour but stronger on dilution (ca. 2 x œstrone).

Ethinyl œstradiol. 0.1 mg. gave an intense orange fluorescence. Intensity: 50 to 100 x œstrone. Two tablets (each 0.05 mg.) crushed and extracted with ether gave a similar reaction when applied to the evaporated ether extract.

Ethisterone (ethinyl testosterone). The reaction produced a dichroic (green-violet) solution in acetic and phosphoric acids, turning to deep red in 1 hour. Dilution with acetic acid gave a red solution with an intense peach coloured fluorescence (50 to 100 x œstrone). Tablets (5 mg.) gave this reaction without extraction.

Methyl testosterone. 1 mg. gave a strong yellow fluorescence (ca. 10 x œstrone). Tablets gave the same result without extraction using approximately one-fifth of one tablet (5 mg.)

œstradiol. An immediate light green fluorescence stronger than œstrone and unchanged after 1 hour was produced by 0.1 to 1 mg. Dilution with acetic acid caused partial quenching.

œstradiol dipropionate. 0.1 to 1 mg. gave no reaction in the cold; heating at 100°C. for 5 minutes initiated a reaction similar to that of œstradiol.

œstradiol monobenzoate. 0.1 to 1 mg. gave no reaction in the cold but behaved similarly to the dipropionate after heating at 100°C. for 10 minutes. The behaviour of the dipropionate and the monobenzoate indicated that

LETTERS TO THE EDITOR

hydrolysis was occurring at 100°C. and also that an OH group in the 3-position is necessary for the production of fluorescence.

Œstrone.—A green fluorescence was produced after 1 minute. After extraction with ether followed by evaporation of the ether extract the residue from tablets of œstrone gave the same reaction.

Progesterone. The reaction produced a weak blue fluorescence; 5 to 10 mg. quantities were required to make the test effective.

Testosterone propionate. This gave no reaction in the cold. On heating at 100°C. for 2 minutes and standing for 1 hour, dilution with acetic acid produced a fairly strong yellow fluorescence. Heating for 5 minutes following a similar procedure gave a red colour with an orange-yellow fluorescence, fairly strong but relatively weaker than œstrone.

SYNTHETIC HORMONES.

Dienœstrol. After dissolving 0.1 mg. in 0.2 ml. of glacial acetic acid, adding 1.8 ml. of 85 per cent. phosphoric acid, allowing to stand for 1 hour and then heating for 1 hour at 100°C. followed by dilution with 3 ml. of acetic acid, a purple colour with an intense but rather unstable violet fluorescence was produced, as described by Boscott. This reaction could be applied to dienœstrol extracted from crushed tablets with ether. Hexœstrol and stilbœstrol gave no reaction to this test.

With the exception of hexœstrol and stilbœstrol all the hormones mentioned can be distinguished when in tablet form by means of the colour and fluorescence reactions described, using, when necessary, ether extraction to separate the hormone from interfering tablet material. Owing to the low intensity of some of the fluorescence effects produced, comparison of an unknown fluorescence with fluorescences produced by known hormones when treated similarly should be used to obtain a reliable fluorescence identification.

Finkelstein, Hestrin and Koch postulated that the production of a fluorescence depended on the presence of a conjugated double bond system and on the position of polar groups. The present results have indicated the importance of a free OH group in the 3-position and have shown that a 17-ethinyl group confers intense fluorescence activity.

Preliminary work on the application of the techniques described to the identification and possible estimation of steroid and synthetic hormones in oily solutions for injection has revealed difficulties which necessitate further study.

We desire to thank the Directors of The British Drug Houses, Ltd., for permission to publish these results.

Analytical Laboratory,
The British Drug Houses, Ltd., London, N.1.
December 20, 1948.

R. G. STUART.
R. E. STUCKEY.

REFERENCES

1. Boscott, *Nature*, 1948, 162, 577.
2. Finkelstein, Hestrin and Koch, *Proc. Soc. exp. Biol., N.Y.*, 1947, 64, 64.
3. Cocking, *Analyst*, 1943, 68, 144.

CORRECTION.—No. 1, p. 61, line 2, for 500 read 550

SCIENTIFIC MEETING

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND THERAPEUTIC ACTIVITY

BY PROFESSOR W. H. LINNELL, D.Sc., Ph.D., F.R.I.C.

Summary of a paper delivered before the Bristol and South-Western Counties Section of the Royal Institute of Chemistry at the University, Bristol, on January 13, 1948

ARGUMENTS whether the physical properties or the chemical properties of any active substance are the more important are largely a waste of time, as both are linked together *via* the molecular structure. The physical properties must be such as will ensure the substance arriving at the seat of action, but once this is attained some "molecular fit" appears to be necessary for the required activity. In fact, some of Pauling's work gives scientific backing to the simile of the lock and key advanced by Fisher.

During comparatively recent times the new conception has been advanced that, in certain attacks on invading organisms, the remedial compound exerts its activity by interference with an essential metabolite. The names of Woods and Fildes are prominent in this respect, the best example being that of the sulphonamides, for which the essential metabolite is *p*-aminobenzoic acid. Progress along this route is to be expected, since it gives a definite direction to research. Difficulty will be encountered from the fact that most cells, whether they belong to an invading organism or the host, are very similar in their essential requirements. Though immediate practical results in the introduction of new substances to medicine will in the near future, in all probability, result from the empirical method, fundamental information will have to take into account the biochemical aspect.

The acridine antiseptics and the synthetic oestrogens provide examples for discussion. During the 1914-18 war the acriflavine type of substance was established as being of great importance in the treatment of wounds, since compounds of this class have the important property of being as active *in vivo* as *in vitro*. In 1935 the two compounds available were both derivatives of 2:8-diaminoacridine, and the preparation of all the isomeric mono and diamino derivatives of the acridine nucleus and comparison of their potency was undertaken. It was found that the 2 and 5 positions were the most active, but that the 5 position appeared to induce increased toxicity. Whenever a 1 (or 8) amino substitution appeared the compound was entirely inactive, a result which has been explained *via* hydrogen bonding. The 2:7-derivative appeared to possess the best properties of a compound of this type in that, although it exhibited a high toxicity against the invading organisms, it was only very slightly toxic against the host. The work of Manifold and Russell on the use of this compound with brain tissue gives support. Albert has since carried this work farther, and claims that for an acridine derivative to be active its *pK* as a base should be above a certain value, and also that the possibility of tautomerism within the molecule concerning the nitrogen grouping might be of importance. A recent preparation, as yet not published, of 1-dimethylaminoacridine, which cannot give rise to hydrogen bonding, and has a *pK* above the minimum as suggested by Albert, but which is inactive, suggests that the last word has not been said in this respect.

NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Anaxeryl.* The active ingredients of this ointment are dioxyanthranol 0.22 per cent., ichthyol 0.85 per cent., balsam of Peru 1.00 per cent., salicylic acid 0.30 per cent., resorcin 0.20 per cent., and birch tar oil 0.30 per cent. It is indicated in psoriasis, persistent dry eczema, lichen planus and various mycotic infections. Anaxeryl ointment may be applied daily to any part of the body. It is supplied in 40 g. tubes. A. O.

Chloroquine Diphosphate. (*New and Non-official Remedies, J. Amer. med. Ass., 1948, 136, 1049.*) Chloroquine diphosphate is 7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline diphosphate, $C_{18}H_{22}O_8N_3ClP_2$; mol. wt. 515.88. It occurs as a white crystalline powder, m.pt. 193°C. to 195°C. , or for a second form of chloroquine diphosphate the m.pt. is 215°C. to 218°C. , readily soluble in water, almost insoluble in alcohol, benzene, chloroform and in ether; a 1 per cent. aqueous solution has pH about 4.5. It has a bitter taste. When dried *in vacuo* over phosphorus pentoxide at room temperature for 48 hours, it loses not more than 2 per cent. of its weight. When a few drops of ammonium molybdate solution are added to 50 mg. dissolved in 3 ml. of water, a white precipitate is produced immediately. On adding 5 ml. of a saturated aqueous solution of picric acid to 20 ml. of a 0.1 per cent. aqueous solution of chloroquine diphosphate, a yellow precipitate is immediately produced, which melts, after washing and drying, between 205°C. and 210°C. (Caution is required for this test!) When 50 ml. of a 0.5 per cent. solution, made alkaline with 1 ml. of strong ammonia solution, is extracted with two quantities, each of 30 ml. of cyclohexane, and evaporated to dryness and allowed to crystallise in a vacuum desiccator over phosphorus pentoxide, the crystals obtained have m.pt. 87°C. to 90°C. *Assay for phosphorus*—50 ml. of a 1.5 per cent. acid solution of bismuth subnitrate is added to 0.2 g., accurately weighed, dissolved in 50 ml. of water, the mixture is digested for 2 hours on a steam-bath, and filtered through a Gooch crucible; the precipitate is washed with dilute nitric acid (2 ml. in 100 ml.), water, alcohol and finally ether, and then dried for 2 hours at 100°C. and weighed. The phosphorus content is not less than 11.8 per cent. and not more than 12.25 per cent. *Assay for chloroquine diphosphate*—0.2 g. accurately weighed, is dissolved in 50 ml. of water, and the solution, made alkaline with 5 ml. of ammonia solution, is extracted with successive quantities of 25, 20, 15, 10 and 10 ml. of ether. The combined ether extracts are filtered and evaporated and the residue, after drying at 100°C. for 30 minutes, is not less than 98 per cent. and not more than the equivalent of 102 per cent. Chloroquine diphosphate is highly active against the erythrocytic forms of *Plasmodium vivax* and *P. falciparum* and is said to have about 3 times the activity of mepacrine against these organisms. It suppresses acute attacks of malaria but is not a prophylactic agent. It is administered by mouth, before or after meals; for suppression of vivax infection 0.5 g. at weekly intervals is recommended; for treatment of acute attacks of vivax or falciparum malaria an initial dose of 1 g. followed by 0.5 g. after 6 to 8 hours and 0.5 g. daily for 2 days is sufficient to terminate the attack. L. H. P.

Depropanex* is a deproteinated pancreatic extract. It is a saline solution of a chemically derived, protein-free, nitrogenous fraction obtained by acid-

alcohol treatment of mammalian pancreas. Depropanex contains no insulin, histamine or acetylcholine, and not more than 2.5 per cent. of solids of which 0.9 per cent. is sodium chloride and 0.5 per cent. is non-protein nitrogen. The pH is adjusted to 6.5 to 6.8. Standardisation is carried out by comparing the effect of the extract on the arterial blood pressure of anaesthetised dogs with that of a standard extract. Each batch is adjusted to contain 10 depressor units per ml. A qualitative test is made by observing the heart-blocking effect in mice. The lowering of arterial blood pressure in urethanised rabbits and atropinised dogs is used as a test for the absence of histamine and acetylcholine. Depropanex has been successfully used in intermittent claudication, especially that associated with occlusive arterial disease, in renal and ureteral colic, spastic ureteritis and dysmenorrhœa. In chronic vascular disease 2 to 3 ml. should be given intramuscularly every other day. For ureteral colic or where there is acute contraction of smooth muscles 3 to 5 ml. should be given. An intramuscular dose of 2 to 4 ml. is recommended for primary dysmenorrhœa. It is not advised that depropanex be injected intravenously. The product is supplied in 10 ml. rubber-capped vials.

A. D. O.

Mycil* is a fungicide, issued in the form of an ointment and a dusting-powder, the active ingredient of which is *p*-chlorophenyl- α -glycerol ether. It is effective against *Epidermophyton floccosum* and the various species of *Tricophyton*, the usual causative organisms of athlete's foot. The ointment is used for treatment of the infection, and the dusting-powder is sprinkled in the socks or shoes as a prophylactic measure.

S. L. W.

Neurinase* is a combination of the active principles of fresh valerian with soluble barbitone. It is claimed that volatile oil containing bornyl isovalerianate, obtained from the fresh rhizome, acts synergetically with the barbiturate. Neurinase is indicated as a hypnotic in insomnia of nervous origin and as a sedative in psycho-neurotic disorders and migraine. It is issued as a solution, containing in a teaspoonful about 2 gr. of soluble barbitone, and in tablets, containing, in each, about 3.3 gr. of soluble barbitone.

S. L. W.

Nitrogen Mustard Hydrochloride,* is di-(2-chloroethyl)methylamine hydrochloride, the nitrogen mustard derivative known in America as bis(β -chloroethyl)amine hydrochloride, or "Bis." It is indicated in cases of Hodgkin's disease which have become resistant to radiation therapy, producing a remission of symptoms, and rendering the case amenable to further X-ray treatment. It does not appear to be more effective therapeutically than radiation therapy in the treatment of lymphosarcoma or lymphatic and myelogenous leukaemia. The results obtained with nitrogen mustard in the treatment of polycythæmia rubra are comparable with those obtained with radio-active phosphorus. It is administered intravenously in a dose of 0.1 mg./kg. of bodyweight for a total of 3 to 6 days; the maximum single dose should not exceed 8 mg. and an interval of 6 to 8 weeks should be allowed between courses of injections. Solutions for injection must be freshly prepared, 10 ml. of a 0.9 per cent. sterile solution of sodium chloride being added to 10 mg. of the salt. Nausea and vomiting and a tendency to hæmorrhage may occur. Extravasation should be avoided. Nitrogen mustard hydrochloride is issued in boxes of 10 vials each containing 10 mg.

S. L. W.

Promizole* is a proprietary brand of 2:4' diamino-5-thiazolylphenyl sulphone, and is used in the oral treatment of leprosy. No claim is made as to the ultimate value in leprosy of promizole given orally, but the therapeutic results so far

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obtained are considered sufficiently encouraging to warrant further clinical study. Doses of 1.5 g., increasing to 6 g., have been given daily for periods of a year or more; the drug is well tolerated. Initial clinical reports indicate that it may also be of value in tuberculosis. Tablets of 0.5 g. are supplied in bottles of 100 and 1,000.

S. L. W.

Prothricin* is an antibiotic nasal decongestant which contains 2.00 per cent. of tyrothricin and 1.5 per cent. of propadrine hydrochloride. The solution is buffered to pH 5.5 to 6.5 and contains 0.002 per cent. of phenylmercuric acetate as a preservative. The shrinking effect of propadrine on the nasal mucosa lasts for 2 hours and causes little or no irritation or side reactions. Prolonged use does not cause the ill-effects of ephedrine on the nasal mucosa. The extensive use of tyrothricin has not given rise to drug sensitivity nor has it caused tissue damage. In this respect it is superior to the sulphonamides. The concentration of tyrothricin in prothricin is effective in the presence of body fluids and tissue exudates against the Gram-positive organisms commonly infecting the respiratory tract and it is moderately effective against the Gram-negative meningococci and gonococci. It is recommended for acute catarrhal rhinitis, rhinosinusitis and ethmoiditis, and the incidence of otitis media and other complications may be reduced by its use. Chronic infections respond less readily. Applied by means of a dropper or spray, prothricin should be used every 15 to 30 minutes or as necessary. Unless there is constant medical supervision it is contra-indicated in heart or thyroid disease, high blood pressure and diabetes; otherwise prothricin seldom causes side effects. The preparation is supplied in dropper-bottles containing 1 fl. oz.

A. D. O.

Scobenol* is a stable emulsion containing 25 per cent. of benzyl benzoate for the treatment of scabies. After bathing and drying, the emulsion is applied over the whole body from the neck downwards with a flat paint brush, and is allowed to dry on. Two such treatments, either on successive days or within a period of 8 days, are sufficient. Scobenol is issued in bottles containing 4 fl. oz., which is sufficient for the complete treatment of an adult.

S. L. W.

T.E.A.B.* is a proprietary form of tetraethylammonium bromide supplied as a 10 per cent. solution for intramuscular or intravenous injection. The tetraethylammonium ion produces a fall in blood pressure, depression of gastro-intestinal motility, pupillary changes, cessation of sweating, dry mouth, and postural hypotension. It should not be administered to patients with low blood pressure or with vasomotor instability, and only with caution to patients with severe hypertension and poor renal function. It is indicated in the treatment of peripheral vascular disease and functional vascular disorders such as Raynaud's syndrome, in thrombo-angiitis obliterans and thrombophlebitis, for the relief of pain in causalgia and in neuralgia following herpes zoster, for the relief of hypertension, and for the alleviation of the pain of peptic ulcer, abdominal cramps and diarrhoea; by distension of the bladder it also relieves pain in certain types of vesical dysfunction. The recommended dosage for intravenous use is 0.2 to 0.5 g. in 10 per cent. solution; intramuscularly the dose should not exceed 20 mg./kg. of body-weight or 15 ml. of the 10 per cent. solution, but usually 5 to 10 ml. is sufficient. Intravenous injection produces an immediate response, but the effect is less prolonged than with the intramuscular injection. It is issued in boxes of 12 and 25 ampoules, each ampoule containing 1 ml. or 5 ml. of 10 per cent. solution.

S. L. W.

NEW REMEDIES

Tivlolac* is a solution of colloidal calcium and vitamin D for injection, subcutaneously or intramuscularly, in allergic states such as urticaria, allergic rhinorrhœa, migraine and asthma, or in vasomotor disorders such as chilblains or angioneurotic œdema. It may also be employed prior to tonsillectomy and dental extraction to reduce capillary hæmorrhage, and it may be given to hasten bony union where this is delayed owing to known calcium deficiency. The average dose is 1 ml. daily for 2 days, followed by a similar dose at 4 to 6-day intervals. Tivlolac contains 0.05 per cent. of colloidal calcium and 5000 I.U. of vitamin D in 1 ml., and is issued in boxes of 6 and 12 ampoules of 1 ml., and in rubber-capped bottles containing 15 and 30 ml.

S. L. W.

Vibelan* tablets contain aneurine hydrochloride 0.5 mg., riboflavine 0.75 mg. and nicotinamide 7.5 mg. in a yeast extract base; it is claimed that the daily administration of 4 tablets supplies the normal adult requirement of these 3 vitamins, the yeast extract providing small unstandardised amounts of other members of the vitamin B group. The use of the tablets is indicated in all vitamin B deficiency states, in seborrhœic dermatoses and some forms of acne, and in patients receiving glucose-saline infusions. Since inactivation of œstrogens by the liver is impaired by vitamin B deficiency, the use of the tablets is also suggested in the treatment of functional uterine hæmorrhage and other hyperœstrogenic states in either sex, including benign prostatic hypertrophy. Vibelan is issued in bottles of 50, 250, and 1,000 tablets.

S. L. W.

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND THERAPEUTIC ACTIVITY (continued from page 132).

With regard to the synthetic œstrogens, it was suggested that the high activity of the stilbœstrol which resulted from the work of Robinson, Dodds *et al* was due to molecular simulation of œstradiol. To probe this contention 3-monohydroxy, 4-monohydroxy and 3:4-dihydroxy- $\alpha\beta$ -diethylstilbene were prepared and tested for œstrogenic activity. The 4-hydroxy compound was very active, an activity which was highly potentiated by the introduction of a second 4-hydroxy group; the 3-hydroxy derivative showed little activity, and the 3:4 dihydroxy showed a lower activity than the 4-monohydroxy compound. These results proved that molecular simulation could not be the whole story, and with any derivative of diethylstilbene a *para*-hydroxy group was of paramount importance. Further exploration of the molecular skeleton, $\alpha\beta$ -diethylstilbene, has resulted in obtaining an activity similar to that of deoxycorticosterone in 4-hydroxy-4- ω -hydroxyaceto- $\alpha\beta$ -diethylstilbene and recently cardiotonic activity in a 4-butenolide.

It will be observed that the examples chosen illustrate the empirical method of attack, but that once an activity has been obtained further work of a systematic character may be prosecuted within the group. Many thousands of different researches, having for their aim the production of something of use in medicine, have produced the comparatively few important synthetic compounds in use to-day, but, although many of the researches lead to a negative result, they none the less contribute to the knowledge of the relationship between chemical structure and therapeutic activity.

REVIEW ARTICLE

SYNTHETIC ŒSTROGENS

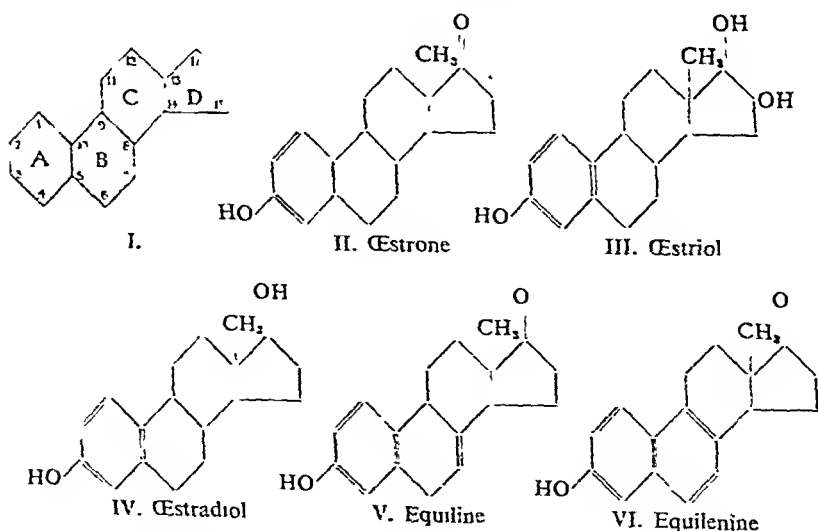
By E. C. DODDS,

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THE field of chemotherapy in general has shown the large numbers of compounds which can be used for the same purposes. One only has to contemplate the development of the so-called "sulpha" drugs to provide an example of this. The first compound produced by Domagk¹ was the complex dye-stuff Prontosil. This substance obtained wide acceptance for the treatment of certain infections, but almost completely disappeared after the brilliant observations of Trefouel, Nitti and Bovet² that in the rabbit the compound was split at the azo linkage, liberating sulphonamide, and that this simple substance itself was active against certain infections. As we know, this was the start of a whole series of drugs of which there must be by now many hundreds on the market.

The same story can be told of the anti-syphilitic remedies, and the same is true of the anti-malarials. The physician wishing to treat either of these conditions has a wide range of compounds to choose from, many of them differing quite fundamentally in constitution, despite the fact that they all bring about the same therapeutic results.



Up to the discovery of synthetic Œstrogens, this phenomenon did not apply to the hormones. There has been only one adrenaline isolated from the suprarenal medulla, only one thyroxine from the thyroid gland and only one insulin from the pancreas.

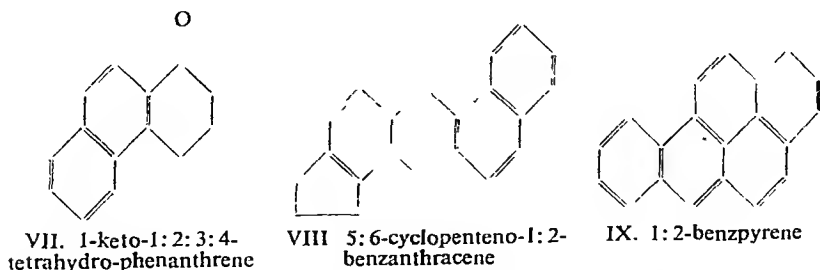
The first indication that there might be a whole series of closely related substances secreted by one endocrine gland came with the isolation of five different oestrogenic substances from the urine of pregnancy, namely, oestrone, oestriol, oestradiol, equiline and equilenine (II to VI). These all have the same qualitative effect, though their quantitative effects are different.

The same story was unfolded concerning the suprarenal cortex. It is now known that a very large number of steroid substances are produced by the cortex of the suprarenal gland, differing in constitution and differing considerably in their metabolic action.

CHEMISTRY

Up to the present time the basic structure of the cyclopenteno-phenanthrene ring system (I) has been found indispensable for androgenic, progestational and adreno-cortical action. In the case of oestrogens it has been found possible to break away from this structure.

It is only necessary to review very briefly the work leading to the synthesis of stilboestrol and its allied compounds. Experiments were begun in the Courtauld Institute about 1930 with the object of seeing how far it was possible to change the molecule of oestrogenic substances without destroying the biological activity. As all naturally-occurring oestrogens contain the phenanthrene system as part of the nucleus, a number of phenanthrene derivatives were prepared and tested by the vaginal smear method (Stockard and Papanicolaou³) on ovariectomised rats. In 1933, the substance 1-keto-1:2:3:4-tetrahydro-phenanthrene (Cook, Dodds and Hewett⁴) was found to be active in rats at a dose level of 100 mg./rat (VII). At the same time a certain similarity was noticed between the microscopic appearance of the cells of the vagina under the influence of oestrogens and the proliferation caused by the painting of carcinogenic hydrocarbons on the skin. Two of the most potent carcinogenic hydrocarbons, 5:6-cyclopenteno-1:2-benzanthracene (VIII) and 1:2-benzpyrene (IX) were tested and found to have definite,

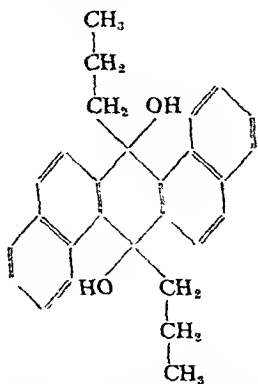


though slight oestrogenic activity (Cook and Dodds⁵). Moreover, it was found that by introducing groups in the 9:10-position of dibenzanthracene this could be converted into quite a powerful oestrogen. A series of 9:10-dihydroxy-9:10-dialkyl-1:2:5:6-dibenzanthracenes was specially

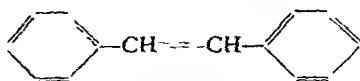
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investigated, and the di-*n*-propyl substituent (X) was found to be active in a dose of 25 μ g. in the rat (Cook, Dodds, Hewett and Lawson⁶).

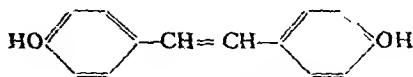
At this point it was decided to see whether the phenanthrene nucleus could be dispensed with and a series of compounds was made, the aim always being to find the simplest possible substance with the highest Œstrogenic activity. Considerable activity was shown by certain compounds with only two benzene rings, particularly by stilbene (XI) and 4:4'-dihydroxystilbene (XII) (Dodds and Lawson⁷). An attempt was then made to "drop" one of the rings and the compound anol. *p*-hydroxypropenylbenzene (XIII), was tested. This appeared to be very highly active (Dodds and Lawson⁸), but when other workers attempted to repeat the observation, considerable variation was found in the different batches of anol, some having only very slight activity. The conclusion was that the activity in some batches of anol was due to



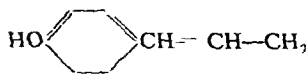
X. 9:10-Dihydroxy-9:10-di-*n*-propyl-1:2:5:6-dibenanthracene



XI. Stilbene



XII. 4:4'-Dihydroxystilbene



XIII. Anol

a contaminant, probably a dimeride of anol. The unsymmetrical dimeride, di-anol (XIV), was tested, but though active, it was not sufficiently so to account for the high activity of some of the batches of anol (Campbell, Dodds and Lawson⁹). The other possibility was the symmetrical dimeride, 4:4'-dihydroxy- α : β -diethyl stilbene, later known as stilbæstrol (XV). This compound was synthesised by a combined team from Sir Robert Robinson's Department at Oxford and from the Courtauld Institute. When tested on rats by the vaginal smear method this was found to be the most powerful Œstrogenic substance then known (Dodds, Golberg, Lawson and Robinson¹⁰). At the same time it was found possible to isolate another compound from the residue remaining from the anol crystallisation, and this compound was later known as hexœstrol (XVI) (Campbell, Dodds and Lawson¹¹). A further compound, dienœstrol (XVII), was made a few months later (Dodds, Golberg, Lawson and Robinson¹²).

These compounds have now been used, particularly for the treatment of menopausal symptoms, for nearly ten years, and have been found to

replace the naturally-occurring œstrogens in every way, with the additional advantage that they are active by mouth. They have also been used since the publication by Huggins¹³ of his observations on carcinoma of the prostate for the treatment of this condition, and have proved to be of great benefit in a large proportion of cases.

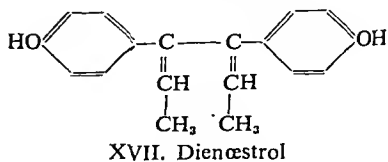
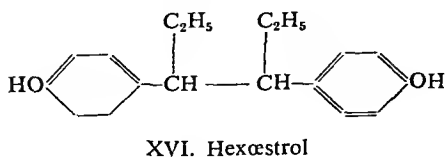
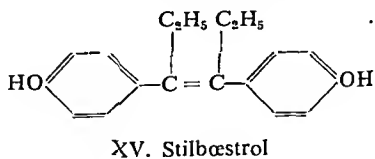
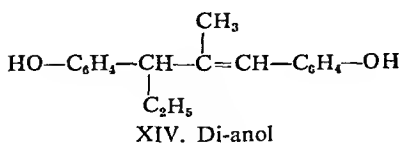
With the establishment of stilbœstrol, hexœstrol and dienœstrol as therapeutic agents, it looked as if the interest in this particular field had more or less come to an end. Recently, however, there have been a number of developments of entirely new synthetic œstrogens, and it is mainly with these that the present account is concerned.

In the first instance, we must abandon the use of the term "synthetic œstrogen," as pointed out recently by Horeau¹⁴. The synthesis of œstrone has now been effected, and therefore the naturally-occurring hormone could also be included under the heading of synthetic œstrogens. For the stilbœstrol type of substance it is better to employ the term "artificial œstrogens" in the future.

The total synthesis of œstrone was effected by Anner and Miescher¹⁵. Whilst this is of great theoretical importance, it would appear very unlikely that the synthetic product will ever compete with the production from natural sources. The natural œstrogens are prepared commercially either from the urine of certain pregnant animals, notably the mare, or from cholesterol by a degradation synthesis.

The fact that there were available three artificial œstrogens for use in therapeutics did not hinder the attempts to find others, since it was hoped by this means to find some clue as to the reason for the œstrogenic activity shown by substances with a constitution far different from that of the natural product.

Recently, clinical interest has been shown in a substance produced by Inhoffen and Hohlweg¹⁶ as long ago as 1938. These workers showed

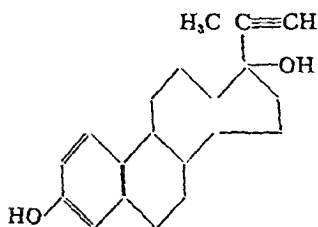


that it was possible to introduce an ethylenic linkage on to the 17-carbon atom in œstradiol. The resulting compound was called ethinyl œstradiol (XVIII). This derivative of the naturally-occurring substance was found to be active by mouth, but it was also stated to suffer from the same

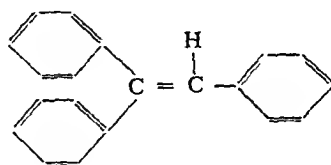
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disadvantages with regard to the production of side-reactions as stilbœstrol. There are no figures available to show the comparative potency of this substance as compared with the others, on laboratory animals, and therefore it will merely be referred to from the clinical point of view.

Attempts to produce substances of the same degree of activity as the stilbœstrol series have not been particularly successful. The activity of diphenylethylene was shown to be definite, but slight. Robson and his colleagues¹⁷ studied the activity of triphenylethylene (XIX) and have shown that derivatives in this series have activity, but again on a much lower plane than the stilbœstrol series. Robson and his colleagues^{18,19} also made some interesting observations on halogen substituted derivatives of triphenylethylene. These substances have not aroused the interest of clinicians.



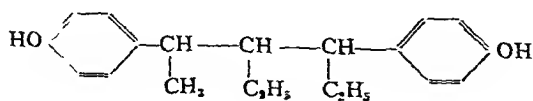
XVIII. Ethinyl œstradiol



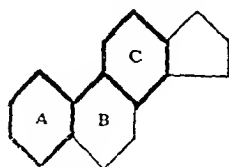
XIX. Triphenylethylene

Another modification of the stilbœstrol type of molecule was made by Blanchard and his colleagues^{20,21} in the synthesis of octofollin, 2, 4-di(*p*-hydroxyphenyl)-3-ethyl hexane (XX). This is a derivative of hexane and the general resemblance to the stilbœstrol formula can be seen by comparing the formulæ. This substance is considerably less active than those of the stilbœstrol series, but it has been offered commercially and there are references to its activity in the human subject (Jaeger²²).

In an attempt to explain the activity of synthetic œstrogens the author suggested (Dodds²³) that some of the substances showing œstrogenic activity might be regarded as stages in the disintegration of the cyclopenteno-phenanthrene nucleus. For example, the activity of the diphenyl series might be explained by the opening of ring B in the manner shown in the diagram (XXI). With the discovery of stilbœstrol, however, this hypothesis was rather neglected, but recently it has been revived in a



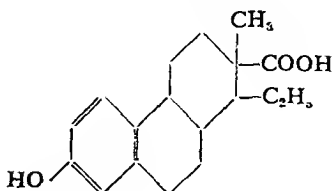
XX. Octofollin



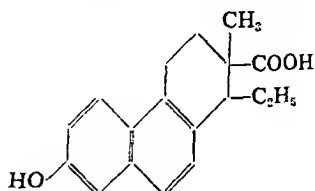
XXI.

very definite form, first by the striking work of Miescher and his colleagues, and later by Horeau and Jacques^{24,25,26,27}.

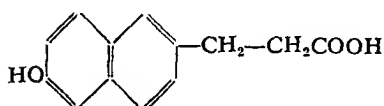
In 1933 Doisy and his colleagues^{28,29} showed that it was possible to produce a very highly active substance from œstradiol by an oxidation process, but they failed to identify the compound so produced. The suggestion that highly active substances could be obtained in this manner suggested to Miescher and his colleagues that the disintegration of the œstrone molecule might produce substances of considerable activity. He therefore synthesised a number of compounds which corresponded to the œstrone molecule with the 5-membered ring opened. Two of these substances have shown great activity. These are referred to as doisylnolic



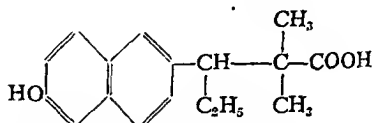
XXII. Doisylnolic Acid



XXIII. bisDehydrodoisylnolic Acid



XXIV. Allenolic Acid



XXV. Dimethylethylallenolic Acid

acid and bisdehydrodoisylnolic acid respectively (Miescher^{24,25}) (XXII, XXIII). There have been extensive clinical trials with the 7-methyl derivative of bisdehydro-doisylnolic acid. Miescher and his colleagues have published a number of papers on the synthesis of this compound and have also described²⁶ a shortening of the synthetic process, but even with this advantage the method of production is infinitely more costly than that of the simpler stilbene derivatives such as stilbœstrol.

Following up the disintegration idea still further, Horeau and Jacques²⁷ synthesised compounds which correspond to doisylnolic acid with the 6-membered ring C opened. This yields a series of naphthalene derivatives, some of which have shown considerable activity. The parent substance has been called allenolic acid (XXIV) and the most active member of the series is dimethylethylallenolic acid (XXV), sometimes referred to as the Horeau acid.

BIOLOGICAL ACTIVITY

The biological standardisation of sex hormones and particularly of œstrogens has always presented very great difficulties. The œstrus reaction in the ovariectomised rat or mouse can only be treated quantitatively on a statistical basis, and the method proposed by Coward and Burn³⁰ is still the basis of all methods of standardisation. This in brief consists in an estimation of the amount of material required to produce full œstrus response in 50 per cent. of a group of ovariectomised animals. According to the size of the group, so vary the reproducibility and

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accuracy of the result. To obtain a reproducibility of 20 per cent. some 20 animals must be used, and in order to get down to 10 per cent. 100 will be required. It can be seen that such a standardisation is very laborious, and in order to make the results of one laboratory comparable with those of another the League of Nations Committee on Biological Standards introduced the international standards of œstrone and œstradiol some years before the war. With the isolation and characterisation of the pure compounds, the difficulties of standardisation very largely disappeared and the dubious use of "rat units" fortunately disappeared from the literature.

The introduction of synthetic œstrogens raises a whole series of new difficulties, and the comparison of the potency of the various synthetic and artificial œstrogens becomes an impossibility. In the first instance, it will be remembered that the potency of an œstrogenic substance depends not only on the actual weight of material administered, but on the length of time over which the administration is spread. By and large one can say that the more one fractionates the dose, the greater will be the potency shown. One of the great difficulties with the synthetic œstrogens is their rate of absorption and destruction in the animal body. It is therefore very difficult to compare on any sound basis the activity of, say, stilbœstrol as against œstradiol. Again, the sensitivity of animals varies from laboratory to laboratory, and therefore it is impossible to compare potencies arrived at in one laboratory with those of another. In the Courtauld Institute a method of standardisation has been worked out, using ovariectomised rats and fractionated injections in sesame oil. By the use of this method it has been possible to arrive at the relative potency of the various synthetic substances which led to stilbœstrol. The results are only comparable in the one institution, and therefore it is not proposed here to make any suggestion that the potencies given are in any way absolute. By our method the following Table gives the potency of the synthetic œstrogens mentioned:

<i>Substance</i>	<i>Dose per Rat</i>
Stilbœstrol	0.3 to 0.4 μ g.
Hexœstrol	0.2 μ g.
Dienœstrol	0.4 μ g.
*7-methyl-bisdehydro-doisyonic acid	0.5 μ g.
Dimethylethylallenolic acid	3.0 to 4.0 μ g.

* This is the figure obtained for the racemic compound. Miescher²¹ has resolved this and has found that the dextro (+) compound is active in rats by single subcutaneous injection in oil in dose of 10.0 μ g., whereas the lævo (−) compound is active in rats in a dose of 0.05 μ g.

From this table it can be seen that by the methods employed, hexœstrol is the most potent substance of the series when administered by subcutaneous injection in oil. With regard to oral administration, it would

appear that in the rat the most potent substance is 7-methyl-bisdehydrodoisynolic acid, with stilbæstrol occupying second place.

CLINICAL ACTIVITY

The earliest tests of stilbæstrol were made under the ægis of the Medical Research Council in 1939 (Bishop, Boycott and Zuckerman³¹, Winterion and MacGregor³²). Since that time a vast literature has accumulated describing the testing and dosage of the various synthetic œstrogens. Out of this very extensive, and in many cases highly uncritical, literature a number of facts emerge:

1. That the synthetic œstrogens are active in the human subject by mouth, and that they are efficient in the treatment of the various gynæcological disorders.

2. That all products in a varying degree do cause side reactions, varying from slight nausea to, very rarely, severe symptoms such as vomiting, skin rashes, and so forth. By and large the reactions are never so severe as to necessitate the discontinuance of the treatment.

3. Astonishing diversity of opinion occurs on the relative potency and percentage of side reactions in these various compounds. In the first instance, the question of side reactions has been the subject of much speculation. Various groups of workers have claimed that one of the synthetic œstrogens is much less prone to produce side reactions than another, and from this it has been concluded that the toxicity is due to some peculiarity of the molecule. To the present writer this has always seemed an unlikely explanation, and in view of the fact that it is possible to get the same type of side reactions with compounds of such widely different structure as ethinyl œstradiol, doisynolic acid and allenolic acid, stilbæstrol, and so forth, it would appear much more reasonable to suppose that the toxicity is associated with the œstrogenic potency. It is known that the naturally-occurring œstrogens are rapidly destroyed in the body, whereas most of the synthetic œstrogens are excreted in the urine either unchanged in part or in conjugation with glucuronic acid.

It has been usual to assume that the sensitivity of all mammals is roughly the same for œstrogens, but there is now considerable evidence that such is not the case, and that it is most unwise to assume that the human female will react in the same way as the laboratory animals. The difficulty in the past has been the lack of any quantitative work on the subject. It is therefore with very great interest that the paper by Bishop, Kennedy and Wynn-Williams³³ has been received. These authors, recognising the lack of quantitative data, have attempted to standardise the œstrogens on the human subject by using œstrogen withdrawal bleeding as a criterion. If œstrogens are given to a menopausal woman with amenorrhœa, amelioration of the symptoms of the menopause occurs almost immediately. If, after a fortnight or so, treatment is suddenly stopped, a small vaginal hæmorrhage occurs. This has been termed œstrogen withdrawal bleeding. Bishop and his colleagues have used

this as an end-point in their standardisation, and by determining the minimum amount of orally active œstrogen necessary to induce this phenomenon, have been able to place the compounds tested in order of potency. The result was that, of the substances tested, stilbœstrol is the most potent. In view of the extremely important nature of their conclusions, the summary is quoted *in extenso*:

“A method is described for comparing the potency of œstrogens in man. It consists in giving the œstrogen daily by mouth in 14-day courses to amenorrhœic women and recording whether œstrogen withdrawal bleeding takes place.

“The results obtained indicate that dienœstrol is about a quarter, doisynolic acid about a fifth, and hexœstrol about an eighteenth as potent as stilbœstrol.

“Investigation of the incidence of ‘toxicity’ indicates that stilbœstrol is more likely to produce nausea in therapeutic doses than are dienœstrol, doisynolic acid and hexœstrol.

“Reasons are given for choosing this end-point, and for the failure to devise any other suitable method of assessment at different levels of œstrogenic response, such as the relief of menopausal symptoms, the production of an œstrous vaginal smear, and the suppression of lactation.”

This work is of very great interest in that it shows the folly of applying results obtained on animals to the human being. For example, there appears to be little doubt that 7-methyl-bisdehydro-doisylnolic acid is highly potent in the rat and mouse by mouth, yet it appears from the results of Bishop and his colleagues to be relatively impotent in the human female.

Finally, ethinyl œstradiol has been the subject of a number of publications, and there is no doubt that it is able to replace the naturally-occurring œstrogens in the same way as stilbœstrol. A number of papers have appeared in America which show that menopausal symptoms can generally be controlled by daily doses of 0.05 to about 0.3 mg. (Wiesbader and Fillet³⁴, Groper and Biskind³⁵, Salmon *et al.*³⁶). Birnberg *et al.*³⁷ have used ethinyl œstradiol with success for the treatment of the menopause and of amenorrhœa, for the suppression of lactation and for the induction or hastening of labour. Ethinyl œstradiol can also be used, like other œstrogens, for the treatment of carcinoma of the prostate (McCrea³⁸). Some papers have appeared in which the potency of ethinyl œstradiol is compared with that of other œstrogens, natural and artificial. Harding³⁹ in a series of 47 cases used ethinyl œstradiol and other œstrogens to treat hypo-ovarian symptoms. Ethinyl œstradiol was shown to be the most active of the substances used, but like stilbœstrol was liable to cause “mild toxic reactions.” Jeffcoate *et al.*⁴⁰ have compared ethinyl œstradiol with other œstrogens on its power to suppress lactation. By this criterion it is also shown to be the most

active. However, as pointed out by Bishop *et al.*³³, this is "an unsuitable method for the clinical assessment of oestrogens," and it is not one that lends itself to quantitative consideration. Finally, Soule⁴¹ has compared ethinyl oestradiol, using oestrogen withdrawal bleeding as the end-point, with stilboestrol, α -oestradiol and "mixed oestrogens." The various oestrogens were only tested on 1 patient, so the results can hardly be considered as statistically significant, but it was shown in this case that ethinyl oestradiol was the most active, producing oestrogen withdrawal bleeding with a dose of 0.05 mg. per day for 21 days, as against a dose of 4 mg. of stilboestrol for 13 days. Both these substances caused nausea when used in effective dosage.

FUTURE RESEARCH

The success obtained in the field of synthetic oestrogens leads one to speculate as to future possibilities in the extension of research. Whilst it is always unwise to prophesy, there would appear to be two main lines of work.

Firstly, is it possible to synthesise compounds with a more selective action on the various tissues acted upon by oestrogens? As Parkes⁴² has pointed out, the word oestrogen is *rather an unfortunate one, since it focusses attention on only one aspect of these compounds' activities, namely, the production of oestrus changes in the vagina.* He has suggested that the term "gynæcogenic" would be better, as this would include all the various activities associated with oestrogenic power, such as development of secondary sexual characteristics, action upon the uterus, breast and anterior lobe of the pituitary. Many have speculated as to whether it would be possible to synthesise a substance with selective action on the anterior lobe of the pituitary, whilst at the same time having little action on the breast, uterus and so on. The advantage of such a compound in the treatment of carcinoma of the prostate is obvious. It is the writer's opinion that there is no evidence that such compound could be found. Experience suggests that these compounds act in their entirety, and that it is not possible to segregate or separate the various actions. In other words, the results are due to oestrogenic activity *per se*.

The second line of speculation is whether it will be possible in the future to make synthetic analogues of the other steroid hormones. In other words, would it be possible to produce a compound with, let us say, androgenic activity, which bears no more resemblance to testosterone than does stilboestrol to oestradiol. Whilst on general grounds it would seem possible that such compounds could be produced, until one has actually been synthesised and its action demonstrated it is obviously idle to speculate.

In conclusion it may be stated that the clinician has a wide selection of artificial oestrogens from which to choose to treat his patients by the oral route. Again, consideration of the evidence would seem to indicate that there is little to choose between any of these substances, and that they are likely to produce side reactions in direct proportion to their oestrogenic potency.

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RESEARCH PAPERS

SYNTHETIC COMPOUNDS RELATED TO THE CARDIAC GLYCOSIDES

PART I—*p*-HYDROXYPHENYL- $\Delta\alpha$: β -BUTENOLIDE GLUCOSIDE

BY W. H. LINNELL AND F. SAID

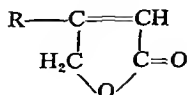
From the Pharmaceutical Chemistry Research Laboratories, the School of Pharmacy, University of London

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INVESTIGATIONS on the natural cardiac glycosides have revealed that the unsaturated lactone ring in the side chain of their aglucones is indispensable for the specific action on the heart. Thus Elderfield¹, Ruzicka², Reichsrein³ and others have been engaged in the preparation of unsaturated lactones related to the natural aglucones, but the compounds they obtained did not show cardiotoxic activity⁴. Comparing the potencies of some cardiac glycosides with those of their aglucones Chen⁵ made it clear that glycosidal combination enhances the cardiotoxic potency up to 12 times. So, whilst a synthetic aglucone of relatively small activity might show no activity whatsoever during pharmacological tests, it is much less likely that the potential cardiotoxic activity of such a compound would be overlooked if it was examined in glycosidal form.

Some special monosaccharides have been found in nature only in combination with the cardiac aglucones, and might be supposed to have an optimum effect upon the activity of the aglucones with which they are combined. However, synthetic glucosides of strophanthidin, digitoxin and digoxin⁶ possess a greater activity than the natural glycosides. Hence no specificity may be expected from the sugar fragment of the molecule. Certain comparatively simple molecules of the substituted butenolide structure have been prepared and examined for cardiotoxic activity. These substances are shown in Table I.

TABLE I



R = CH₃⁷; -C₂H₅; -CH₂.CH₂.CH₃
 = -C₆H₅; -C₆H₄(OH) [1:4]; -C₆H₄(OH) [1:3]⁸; -C₆H₄(OCH₃) [1:3]⁹
 = cyclohexyl; 2-chloro-cyclohexyl; *cis* and *trans* 4-hydroxy
 = cyclohexyl¹⁰; 3:4-dihydroxycyclohexyl¹¹
 = Δ^3 -cyclohexenyl¹¹
 = cyclopentanyl¹²
 = α -naphthyl; β -naphthyl; 6-hydroxy- β -naphthyl,
 6-methoxy-2-naphthyl; decahydro- β -naphthyl¹
 = 1-indanyl³

Although the methyl and the β -naphthyl derivatives showed a minute reaction in frogs (active at a dose of 2 mg./g.);* all the other substances were inactive. This level of activity is hardly significant. However, none of the compounds has been converted into glycosides and tested in this form.

For these reasons it was decided to prepare some compounds which possess the characteristic unsaturated lactone ring in conjugation with simple hydroxylated carbon skeletons, and convert them into their respective glucosides before pharmacological testing. The first member of this series, *p*-hydroxyphenyl- $\Delta\alpha:\beta$ -butenolide glucoside, was obtained by the action of acetobromoglucose on *p*-hydroxyphenyl- $\Delta\alpha:\beta$ -butenolide⁶ and subsequent deacetylation of the tetra-acetylglucoside thus obtained by means of barium methoxide. The glucoside was obtained as a white microcrystalline powder melting at 208° to 209°C.; it had a faint bitter taste and was very hygroscopic and freely soluble in alcohol and in water. It gave a positive Legal's test and was hydrolysed on boiling in water. The analytical figures were in accord with those required.

Pure acetobromoglucose necessary for the reaction was obtained in good yields by a modification of the process usually used for its preparation¹³.

Neither the pure aglucone nor the glucoside showed any cardiotonic activity. The tetra-acetylglucoside was insoluble in ordinary solvents and thus could not be examined.

EXPERIMENTAL

Acetobromoglucose. The following method was found to be better than the normal method for the preparation of the compound.

Glucose penta-acetate (10 g.) was covered with commercial 50 per cent. solution of hydrogen bromide in glacial acetic acid (20 ml.) at 0°C. The mixture was left at room temperature overnight, then gradually poured with stirring into a large excess of ice-cold water. The acetobromoglucose, which separated as a white crystalline mass, was filtered, washed with ice-cold water and dissolved in warm methyl alcohol, and the solution kept in a refrigerator for 1 hour. The compound separated out in long colourless needles, which were filtered and recrystallised from isopropyl ether. Yield 95 to 98 per cent.; m.pt. 91°C.

p-O-Tetra-acetylglucosidoxyphenyl- $\Delta\alpha:\beta$ -butenolide. *p*-Hydroxyphenyl- $\Delta\alpha:\beta$ -butenolide⁶ (1 g.) dissolved in 2 per cent. aqueous sodium hydroxide (10 ml.) was added to a solution of acetobromoglucose (2.5 g.) in acetone (10 ml.) and the mixture shaken for 5 hours. 2 per cent. sodium hydroxide solution (15 ml.) and acetobromoglucose (2 g.) were added and the mixture shaken for a further 12 hours. The precipitate that formed was filtered, washed with 10 per cent. sodium hydroxide solution and crystallised from alcohol. It formed colourless shining plates, m.pt. 195° to 195.5°C. Yield 36 per cent. Found; C, 56.72; H, 5.01; $C_{24}H_{26}O_{12}$ requires C, 56.89; H, 5.10 per cent.

p-Hydroxyphenyl- $\Delta^{\alpha}:\beta$ -butenolide glucoside. 2N Barium methoxide¹⁴ (0.1 ml.) was added to a suspension of the glucoside acetate (0.4 g.) in methyl alcohol (40 ml.) and the mixture kept at ordinary temperature in a stoppered flask for 5 days. The solution was exactly neutralised by the addition of 0.5N sulphuric acid (1 ml.) and, after allowing to stand for half an hour, the precipitate was filtered off. The filtrate was evaporated to dryness under reduced pressure and the residue dissolved in methyl alcohol; on adding dry ether to the solution a white precipitate was thrown down. The precipitate was rapidly filtered, washed with ether followed by light petroleum and then kept in a vacuum desiccator until dry. The glucoside formed a white microcrystalline powder, m.pt. 208° to 209°C. Yield 90 per cent. Found: C, 54.5; H, 5.64 per cent.; $C_{16}H_{16}O_8$ requires: C, 56.5; H, 5.4 per cent.

The aglucone, glucoside acetate and glucoside gave a positive Legal's test.

Attempts to form the glucoside acetate by shaking in presence of active silver oxide in different solvents alone¹⁵ and in presence of pyridine¹⁶ gave amorphous brown products which could not be purified.

Thanks are due to Professor Buttle and Dr. Dyer, of the Pharmacological Department of this School, for the physiological testing of these compounds.

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SYNTHETIC COMPOUNDS RELATED TO THE CARDIAC GLYCOSIDES

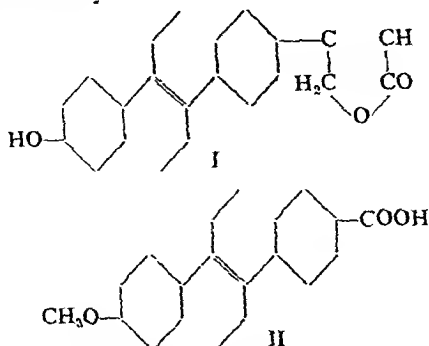
PART II

BY W. H. LINNELL AND F. SAID

From the Pharmaceutical Chemistry Research Laboratories, the School of Pharmacy, University of London

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IN Part I of this series the *p*-hydroxyphenyl- $\Delta\alpha:\beta$ -butenolide and its β -glucoside were found to have no cardiac activity.¹ The success which has attended the use of the stilbene nucleus in the preparation of synthetic oestrogens naturally encourages the use of this nucleus in other fields of steroid derivatives. For this reason it was decided to attempt the preparation of a 4-hydroxy-4'- $\Delta\alpha:\beta$ -butenolido- $\alpha:\beta$ -diethylstilbene (I) in the hope that this molecule or the glucoside prepared from it would exhibit the desired activity.



Some little difficulty was encountered in the preparation of this compound, but at length its methyl ether was obtained. The starting point of the synthesis was, 4-methoxy- $\alpha:\beta$ -diethylstilbene-4'-carboxylic acid (II) which had been previously obtained in low yields by Jaeger and Robinson² and by Neher and Miescher³, and therefore attempts were made to improve this preparation. The previous authors obtained (II) by the hydrolysis of the corresponding nitrile although difficulties had previously been encountered in the hydrolysis of such stilbene derivatives^{4,5,6}.

This difficulty was surmounted by the hydrolysis of 4-methoxy-4'-cyanodeoxybenzoin² with a mixture of acetic and sulphuric acids, when a good yield of 4-methoxy-4'-carboxy-deoxybenzoin was obtained in colourless needles melting at 223° to 224°C. It was slightly soluble in alcohol, ether and benzene. This acid was converted into its ethyl ester which, was obtained in fine white needles melting at 136° to 137°C. It gave the required analytical figures as did its 2:4-dinitrophenylhydrazone which crystallised from benzene-light petroleum mixture in red scales m.pt. 165° to 166°C.

The above ester was ethylated by means of sodium ethoxide in ethyl alcohol and the 4-methoxy-4'-carbethoxy- α -ethyl-deoxybenzoin obtained

was purified by distillation *in vacuo*;¹ it formed a pale yellow oil boiling at 220° to 223°C./0.2 mm. Hg. pressure. The 2:4-dinitrophenylhydrazone separated as an oil and could not be induced to crystallise. The ester was easily hydrolysed to the free acid which, separated as an oil and solidified into a resinous mass which was subsequently crystallised from benzene-light petroleum mixture in thick needles melting at 125° to 126°C. Both the ester and the acid gave analytical figures according with those theoretically required.

The resulting 4-methoxy-4'-carbethoxy- α -ethyldeoxybenzoin was treated with ethyl magnesium iodide to yield the corresponding tertiary alcohol, but during the final distillation involved in the purification of the isolated product, water was eliminated from the molecule and ethyl 4-methoxy- α : β -diethylstilbene-4'-carboxylate was isolated in good yield and quantitatively hydrolysed to the desired stilbene acid (II). The conversion of this stilbene acid into the corresponding acid chloride was effected without difficulty with thionyl chloride and, without isolation, the product was treated with diazomethane to give the diazoketo derivative which, on warming with glacial acetic acid yielded 4-methoxy-4'- ω -acetoxyacetyl- α : β -diethylstilbene. This compound appeared as a thick oil boiling at 208° to 211°C. (bath temperature)/0.1 mm. Hg. pressure and formed a crystalline 2:4-dinitrophenylhydrazone melting at 232° to 233°C. Both the ketone and its dinitrophenylhydrazone gave the required analytical figures.

The above compound was treated with ethyl bromoacetate in presence of zinc to give the required lactone 4-methoxy- α : β -diethylstilbene- $\Delta\alpha$: β -butenolide in the form of pale microcrystalline powder melting at 94° to 95°C. It gave a positive Legal's test, and the analytical figures accorded with the theoretical requirements.

Unexpected difficulty was encountered in the attempts made to demethylate this stilbene-butenolide and further work is in progress to achieve this end and then to convert the demethylated substance into its glucoside.

Preliminary pharmacological examination, for which we are indebted to Professor Buttle and Dr. Dyer of the Pharmacological Department of this School, indicated that the lethal dose for guinea-pigs was about 200 mg./kg.; the death was characteristic of the cardiac aglucones which suggests that the compound possessed approximately 1/1000 the potency of strophanthidin. This result is encouraging because it is more than probable that the demethylated compound would possess a much higher level of activity. Again the conversion of the compound into its glucoside would in all probability produce a further increase in activity. This work is therefore being continued in order to obtain the glucoside which might be expected to exhibit a reasonable level of activity.

EXPERIMENTAL

4'-Carboxy-4-methoxydeoxybenzoin. 4-Methoxy-4'-cyanodeoxybenzoin² (2.5 g.) was dissolved in glacial acetic acid (20 ml.), sulphuric acid (20 ml.) and water (20 ml.) were added and the mixture boiled under

SYNTHETIC COMPOUNDS RELATED TO THE CARDIAC GLYCOSIDES

reflux for 4 hours. The cooled mixture was diluted with water and filtered, and the precipitate recrystallised from 60 per cent. acetic acid. The acid was obtained in colourless needles. It is slightly soluble in alcohol, ether and benzene, m.pt. 223° to 224°C . Yield 82 to 85 per cent. Found C, 71.0; H, 5.13 per cent.; $\text{C}_{16}\text{H}_{14}\text{O}_4$ requires C, 71.11; H, 5.19 per cent.

Esterification of the acidic group. Hydrogen chloride was passed into a boiling alcoholic solution of the acid (2 g.) for 4 hours. The solution was concentrated under reduced pressure and the ester, which crystallised out, was filtered off and recrystallised from 90 per cent. alcohol. It formed fine white needles, m.pt. 136° to 137°C . Yield 85 per cent. Found C, 71.15; H, 6.14 per cent.; $\text{C}_{18}\text{H}_{18}\text{O}_4$ requires C, 72.50; H, 6.04 per cent. The 2:4-dinitrophenylhydrazone crystallised from benzene-light petroleum mixture as red scales, m.pt. 165° to 166°C . Found C, 60.34; H, 4.00; N, 11.1 per cent. $\text{C}_{24}\text{H}_{22}\text{N}_4\text{O}_7$ requires C, 60.00; H, 4.00; N, 10.2 per cent.

4'-Carbethoxy-4-methoxy- α -ethyldeoxybenzoin. 4'-Carbethoxy-4-methoxydeoxybenzoin (5.5 g.) was mixed with absolute alcohol (50 ml.) and the mixture raised to boiling. A solution of sodium ethoxide (0.5 g. of sodium in 10 ml. of alcohol) was added and after 10 minutes boiling, ethyl iodide (3 g.) was added and the mixture strongly boiled for 10 minutes more. Sodium ethoxide solution (0.25 g. of sodium in 6 ml. of alcohol) was added followed by ethyl iodide (1 g.). After 2 hours refluxing, another addition of sodium ethoxide (0.25 g. of sodium and 6 ml. of alcohol) and ethyl iodide (1 g.) was made and the whole refluxed for 6 hours. To isolate the desired compound, the neutral solution was diluted with water, acidified with dilute sulphuric acid and extracted with ether. The ethereal extract was washed with aqueous sodium carbonate, sodium thiosulphate solution and water respectively, then dried over anhydrous sodium sulphate. The residue remaining after the removal of ether was distilled under reduced pressure. 4'-Carbethoxy-4-methoxy- α -ethyldeoxybenzoin distilled as a pale yellow oil at 220° to 223°C . /0.2 mm. Hg. pressure. Yield 90 per cent. Found C, 73.9; H, 6.91 per cent.; $\text{C}_{20}\text{H}_{22}\text{O}_4$ requires C, 73.6; H, 6.61 per cent.

The 2:4-dinitrophenylhydrazone separated as a semisolid mass which could not be crystallised.

Hydrolysis. The ester (1 g.) was boiled with 10 per cent. sodium hydroxide solution (20 ml.) for 1 hour, when it completely dissolved. Treatment of the clear solution with dilute sulphuric acid produced a milky precipitate, which solidified into a resinous mass. It was crystallised from a mixture of benzene and light petroleum in clusters of thick needles, m.pt. 125° to 126°C . Found C, 71.93; H, 5.77 per cent.; $\text{C}_{18}\text{H}_{18}\text{O}_4$ requires C, 72.50; H, 6.04 per cent.

4'-Carbethoxy-4-methoxy- α,β -diethylstilbene. An ethereal solution of ethyl magnesium iodide prepared from ethyl iodide (17.26 g.) and magnesium turnings (2.23 g.) in ether in the usual way, was added with stirring to a solution of 4'-carbethoxy-4-methoxy- α -ethyldeoxybenzoin

(10 g.) in dry ether (100 ml.), with continual stirring during the addition and for 1 hour more. The mixture, carefully protected from moisture, was left overnight and then refluxed for 2 hours. It was then cooled and decomposed by means of ice and hydrochloric acid; the oil that separated was extracted with ether, the ethereal extract dried over anhydrous magnesium sulphate, and the ether then removed. The residue was distilled *in vacuo* and the fraction boiling at 180° to $183^{\circ}\text{C}/0.2$ mm. Hg. pressure was collected. It formed a colourless thick oil and its solution in carbon tetrachloride decolorised bromine. Found C, 78.4; H, 7.77 per cent.; $\text{C}_{20}\text{H}_{22}\text{O}_3$ requires C, 78.1; H, 7.71 per cent.

4-Methoxy-4'-carboxy- α : β -diethylstilbene. The above ester (5 g.) was boiled with a mixture of alcohol (10 ml.) and 10 per cent. aqueous sodium hydroxide (10 ml.) for 1 hour. On cooling, the sodium salt of the acid crystallised out. It was filtered off and boiled with glacial acetic acid (20 ml.), when 4-methoxy-4'-carboxy- α : β -diethylstilbene separated in colourless needles on cooling, m.pt. 175° to 176°C . Yield 96 per cent.

4-Methoxy-4'- ω -acetoxyacetyl- α : β -diethylstilbene. 4-Methoxy- α : β -diethylstilbene-4'-carboxylic acid (5 g.) was dried by heating on a water-bath under reduced pressure for 2 hours. The dry acid was converted to its acid chloride by refluxing with thionyl chloride (20 g.) for 4 hours. On removal of the excess of thionyl chloride, the acid chloride remained as a semisolid mass, which was dissolved in ether and added at 0°C . to an ethereal solution of diazomethane⁷ prepared from 60 g. of nitrosomethyl urea⁸. The mixture was kept at 0°C . for 1 hour, then at room temperature for 16 hours. Ether was removed under reduced pressure and the crude diazoketone thus obtained, mixed with glacial acetic acid (20 ml.) and heated on a water-bath for 2 hours after which no more nitrogen was evolved. The mixture was cooled, diluted with ether, shaken with water and then with sodium carbonate solution. The ethereal layer was separated and dried over calcium chloride; on removal of the ether, the ketol acetate was left as a thick oil, which was purified by distillation *in vacuo*. It distilled at 208° to 211°C . (bath temperature)/ 0.1 mm. Hg. pressure. Yield 70 per cent. Found C, 74.00; H, 6.8 per cent.; $\text{C}_{27}\text{H}_{26}\text{O}_4$ requires C, 75.4, H, 7.1 per cent.

The 2:4-dinitrophenylhydrazone, crystallised from alcohol in the form of an orange microcrystalline powder, m.pt. 233° to 234°C . Found C, 63.60; H, 5.37; N, 10.01 per cent.; $\text{C}_{20}\text{H}_{16}\text{O}_6\text{N}_4$ requires C, 63.73; H, 5.5; N, 10.25 per cent.

4-Methoxy- α : β -diethylstilbene-4'- $\Delta\alpha$: β '-butenolide. Zinc (2 g.) was added to a solution of 4-methoxy-4'- ω -acetoxyacetyl- α : β -diethylstilbene (3 g.) in benzene (20 ml.) and the mixture raised to boiling on a water-bath. Ethyl bromoacetate (3 g.) in benzene (10 ml.) was then gradually added to the boiling mixture with stirring. After 2 hours refluxing and stirring, the reaction mixture was cooled and decomposed by means of ice and concentrated hydrochloric acid. The benzene layer was separated, washed with water and sodium carbonate solution respectively and dried over anhydrous sodium sulphate. After removing the benzene under

reduced pressure, the residue was heated on a water-bath under reduced pressure for half an hour, then mixed with a 50 per cent. solution of hydrobromic acid in glacial acetic acid and heated again for another half hour, and finally poured into a large volume of ice-cold water. The precipitate that formed was filtered and recrystallised from benzene. It formed pale yellow crystals, m.pt. 94° to 95°C . Yield 24 per cent. Found C, 79.8; H, 6.74 per cent.; $\text{C}_{23}\text{H}_{24}\text{O}_3$ requires C, 79.31; H, 6.92 per cent. The compound gave a positive Legal's test.

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THE HISTOLOGY OF BELLADONNA ROOT

PART IV

THE DIFFERENTIAL VALUE OF THE FIBRE/VESSEL RATIO

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BELLADONNA ROOT is defined in the British Pharmacopœia, 1948, as the root, or root and rootstock, of either *Atropa Belladonna* Linn., or *Atropa acuminata* Royle ex Lindley or a mixture of both species. In the earlier parts of this work, it was noted that the xylem of *A. Belladonna* root consists in the main of cellulosic parenchyma and scattered vessel strands, whereas in *A. acuminata* the cellulosic parenchyma is largely replaced by lignified fibrous tissue. Inspection of transverse sections of the roots of the two species did not indicate any differences in the numbers of vessels per unit area, hence it seemed likely that a differential character could be based on the ratio of the numbers of fibres to the numbers of vessel elements. It was therefore decided to investigate this character on both the whole drug and the powder and to adopt the term "fibre/vessel ratio" to indicate the number of fibres associated with one vessel element. In counting the fibres, it was anticipated that the somewhat similar fibre-tracheids might cause difficulty in their discrimination, and to avoid this they were included in the fibre count if they exhibited elongated simple pits. True tracheids, due to their characteristic elliptical pits, are readily distinguished.

In Part III of this work,¹ it was shown that the vessel index provided a means of differentiating the powdered drugs, but its application to the whole drug was not investigated in detail. It appeared desirable therefore to do this also in order to compare the two characters and to assess their value when combined in a discriminant function.

MATERIALS

Specimens of the whole drug were selected from a number of parcels of commercial material obtained from reputable wholesale druggists between 1940 and 1945, and supplied as either "English Belladonna root" or "Indian Belladonna root." Ten specimens of each variety were selected, ranging in diameter from the narrowest to the widest in the material and including examples at all the stages of xylem development present. The wider of these specimens consisted of both root and rootstock, and the narrower of root only. They were numbered 1 to 10 in decreasing order of diameter, which order corresponded approximately with their age, the widest specimens of either variety being about five years old.

The powdered material consisted of six samples of commercial powder, coded AB1 to AB6, supplied as *A. Belladonna* and five coded Aa1 to Aa5, supplied as "Indian Belladonna." This was the same material as that used for the determination of the vessel index and the cork cell number reported on in Part III of this work,¹ in Table I of which fuller details appeared.

THE HISTOLOGY OF BELLADONNA ROOT. PART IV

A.—THE WHOLE DRUG

Number of observations. Since the fibres are more numerous than the vessel elements, it was found convenient in practice to count the numbers of the latter associated with groups of 10 fibres. To determine the minimum number on which the ratio should be based in order to obtain good agreement between successive results, one root of each species taken at random from the commercial drug was examined and the number of vessel elements associated with a total of 400 fibres determined in each case. The range of successive results based on count of 100 fibres and 200 fibres was respectively about 8 per cent. and 4 per cent. of the mean. Subsequent work was therefore based on a minimum count of 200 fibres.

Variation in the whole drug. The 10 roots of each species were softened by soaking in dilute alcohol and cut into 5 approximately equal lengths, which were numbered 1 to 5 from base to apex or crown. The entire portion, or a longitudinal sector according to the bulk, was disintegrated separately by means of Schultz's maceration fluid, following the method described below. The fibre/vessel ratio was calculated from observations of the number of vessel elements associated with 200 fibres; at the same time, and using the same microscopical preparations, the vessel index 135μ (i.e. the percentage of vessel elements wider than 135μ) was also determined.

SUMMARY OF RESULTS

The values of the fibre/vessel ratio for the whole drug are given in Table I, and of the vessel index 135μ in Table II. The ranges of the positional values of the fibre vessel ratio are: *A. Belladonna*, 0.89 to 1.79 to 4.28 to 7.69, mean 3.04, standard deviation, 1.25; *A. acuminata*, 2.35 to 2.20 to 8.79 to 18.18, mean 5.49, standard deviation 3.29. For the vessel index 135μ , the corresponding ranges are: *A. Belladonna*, 0 to 1.42 to 7.14 to 10.07, mean 4.28, standard deviation 2.86; *A. acuminata*, 6.37 to 8.58 to 22.30 to 27.74, mean 15.44, standard deviation 6.86. The ranges of either character thus show a not inconsiderable overlap so that complete differentiation of the whole drug is not possible by these means. Fisher has shown that, in cases such as this, an im-

TABLE I
FIBRE/VESSEL RATIO—VARIATION IN THE WHOLE DRUG

Species	<i>A. Belladonna</i>						<i>A. acuminata</i>					
	Position					Root Mean	Position					Root Mean
	1	2	3	4	5		1	2	3	4	5	
1	1.79	1.62	1.75	2.27	2.63	1.93	5.26	6.06	6.25	6.90	7.69	6.43
2	2.93	2.90	3.70	3.23	4.76	3.52	8.00	10.53	10.53	12.50	12.50	10.81
3	2.25	3.77	3.70	3.70	4.05	3.50	2.86	3.85	3.85	5.88	5.56	4.40
4	3.77	3.70	4.55	4.38	7.69	4.81	6.35	10.00	11.11	14.29	18.18	12.01
5	0.89	1.35	1.32	1.74	1.90	1.44	2.60	2.35	2.86	4.44	4.03	3.27
6	2.25	2.27	2.70	3.13	2.94	2.66	2.99	2.85	3.12	3.12	3.03	3.03
7	1.55	1.92	2.25	2.47	3.23	2.28	3.74	3.57	2.94	3.57	2.53	3.07
8	3.28	3.51	4.55	4.26	4.55	4.03	3.43	4.00	4.65	5.00	5.71	4.49
9	1.87	2.25	2.99	3.13	3.25	2.70	2.69	3.51	3.57	5.26	5.56	4.10
10	2.60	2.94	3.03	3.03	5.55	3.51	3.03	3.45	3.64	2.86	3.70	3.34
Position Mean	2.23	2.62	3.06	3.13	4.09	3.038	3.96	5.02	5.25	6.38	6.86	5.494

provement may result from the use of a discriminant function X , which is a linear compound of the available measurements, i.e. $X = \lambda_1 x_1 + \lambda_2 x_2$, the constants λ_1 and λ_2 being chosen so as to maximise the ratio of the difference between the specific means to the variance within the species.

TABLE II
135 μ VESSEL INDEX—VARIATION IN THE WHOLE DRUG

Species		<i>A. Belladonna</i>					<i>A. acuminata</i>				
Root No.	Position					Root Mean	Position				Root Mean
	1	2	3	4	5		1	2	3	4	
1	3.85	3.10	2.72	1.19	1.19	2.41	21.38	24.47	26.04	27.11	25.38
2	7.41	8.76	8.42	8.09	8.09	8.15	10.07	10.40	9.75	10.71	10.45
3	6.02	6.72	7.41	8.76	8.76	7.53	10.07	12.28	11.98	14.09	12.68
4	1.96	2.72	3.10	3.85	4.22	3.17	13.79	16.39	16.67	16.94	15.03
5	3.10	3.10	3.10	3.10	3.85	3.25	15.25	16.94	18.57	19.87	18.74
6	0.79	0.79	1.58	1.19	1.96	1.26	20.64	23.31	23.78	24.93	23.61
7	7.06	7.41	8.09	10.07	10.07	8.54	17.76	21.88	23.78	25.81	23.39
8	1.96	1.96	2.72	3.47	3.47	2.72	6.37	6.72	6.72	6.72	6.99
9	4.22	4.58	4.58	5.30	6.02	4.94	9.09	9.75	10.07	10.07	9.88
10	0.40	1.19	0.40	0.00	1.96	0.79	7.06	7.41	8.09	8.76	8.28
Position Mean	3.68	4.03	4.21	4.50	4.96	4.277	13.15	14.96	15.55	16.50	15.439

Accordingly, the discriminant function combining the positional fibre/vessel ratio and the vessel index values in Tables I and II was calculated. The calculation followed the method detailed by Mather², with the result $X = 0.004431x_1 + 0.004760x_2$, where x_1 and x_2 are the vessel index and fibre/vessel ratio values respectively. For convenience in practice, a new function $X^1 = 225.6832X$ was employed, i.e. $X^1 = \text{vessel index} + 1.074 \text{ fibre/vessel ratio}$. The results are given in Table III and discussed later.

B.—THE POWDERED DRUG

Preliminary. Before the fibre/vessel ratio can be determined on the powdered drug, the method as applied to whole roots needs modifying to allow for the presence of broken elements. Accordingly, portions of two samples of commercial powdered Indian belladonna root, Aa1 and Aa2, were disintegrated and used for this preliminary work.

TABLE III
VALUES OF $X^1 = \text{VESSEL INDEX} + 1.074 \text{ FIBRE/VESSEL RATIO}$ FOR THE WHOLE DRUG

Species		<i>A. Belladonna</i>					<i>A. acuminata</i>				
Root No.	Position					Root Mean	Position				Root Mean
	1	2	3	4	5		1	2	3	4	5
1	5.34	4.84	4.60	3.55	4.02	4.47	27.03	30.98	32.75	34.52	32.26
2	10.62	11.88	12.39	11.56	13.20	11.93	18.66	21.71	21.06	24.14	22.07
3	8.44	10.77	11.38	12.73	13.14	11.29	13.14	16.41	16.11	20.41	17.40
4	6.01	6.69	7.99	8.52	12.48	8.34	20.72	27.13	28.60	32.29	27.92
5	4.06	4.55	4.52	4.97	5.89	4.80	18.04	19.46	21.64	24.64	22.25
6	3.21	3.23	4.48	4.55	5.12	4.12	23.85	26.38	27.13	28.28	26.86
7	8.73	9.47	10.51	12.72	13.54	10.99	20.70	25.71	26.94	29.64	26.69
8	5.48	5.73	7.61	8.05	8.36	7.04	9.68	11.02	11.71	12.09	11.81
9	6.23	7.00	7.79	8.66	9.54	7.84	11.88	13.52	13.90	15.72	14.28
10	3.19	4.35	3.71	3.25	8.28	4.56	10.31	11.12	12.00	11.83	11.86
Position Mean	6.13	6.85	7.50	7.86	9.36	7.538	17.40	20.34	21.18	23.36	21.34

THE HISTOLOGY OF BELLADONNA ROOT. PART IV

Three methods of assessing the numbers of whole elements equivalent to the fragmentary ones appeared worthy of trial. These were based on:—

- (1) measurement of the total length of the fragments and division by the mean whole element length of the sample;
- (2) counting the numbers of fragmentary elements and multiplying by a factor equivalent to the ratio of the mean fragment length to the mean whole element length;
- (3) counting the fragmentary elements as whole ones according to a predetermined convention.

Method (1). The mean lengths of the broken elements based on 50 measurements were found to be:—fibre fragments— 227.5μ ; vessel element fragments— 184.5μ . Three slides were examined for each sample, the numbers of whole elements being counted, and the lengths of the broken elements in the same fields determined with the aid of a camera lucida. Results are incorporated in Table IV.

TABLE IV

Sample	<i>A. acuminata.</i> Aa1			<i>A. acuminata.</i> Aa2		
	Fibres	Vessels	Ratio	Fibres	Vessels	Ratio
Length of fragments μ	2158	579		7020	754	
Equivalent number whole	9.5	3.1	3.1	30.9	4.9	6.3
Counted number whole	142	23	6.2	189	30	6.3
Total as whole	151.5	26.1	5.8	219.9	34.9	6.3

Method (2). The three slides of each sample were re-examined and the numbers of whole and broken elements counted. The mean length of the vessel elements has already been recorded for the whole drug³, viz. 252μ , hence the factor by which the number of fragments should be multiplied to estimate the equivalent number of whole elements is $184.5/252=0.732$ which can be approximated to $\frac{3}{4}$. The mean length of the fibres (including fibre-tracheids as described above) was estimated as 450μ , hence the corresponding factor is $227.5/450=\frac{1}{2}$, approximately. Results are incorporated in Table V.

Method (3). For this method, the following convention was adopted. Broken fibres were counted as whole if they tapered towards both ends, otherwise they were ignored. Broken vessel elements were counted as

TABLE V

Sample	<i>A. acuminata.</i> Aa1			<i>A. acuminata.</i> Aa2		
	Fibres	Vessels	Ratio	Fibres	Vessels	Ratio
Number of fragments	394	105		587	125	
Equivalent number whole	197	79	2.5	294	94	3.1
Counted number whole	240	39	6.1	399	68	5.9
Total as whole	437	118	3.7	693	162	4.3

whole if they exhibited portions of the perforation rim at both ends; if visible only at one end, they were counted as half; all other fragments were ignored. The number of such vessel elements associated with 300 such fibres was determined on the three slides of each sample examined previously. The fibre/vessel ratios were:—Sample Aa1, 6.25; Sample Aa2, 6.67.

The relative merits of the three methods were now considered before proceeding with the examination of the powdered drug. The standard by which they may be compared is the value of the fibre/vessel ratio obtained from counts of the whole elements only. This value is reasonably consistent for the first two methods, having regard to the relatively small numbers of elements on which it is based. The results for sample Aa2 by method (1) show that this method is capable of giving consistent results, but the process of measuring the broken elements is tedious and does not recommend itself in practice owing to the time involved. Method (2) does not suffer from the latter disadvantage, but the results do not suggest that an accurate estimate of the fibre/vessel ratio could be obtained in this way. The results obtained by method (3) are consistent with those obtained by counting whole elements only and the process is simple to apply in practice. Moreover, the number of elements that can be observed in a given time is much larger than that possible by method (1), thus reducing variation due to random effects. Having regard to the above considerations, it was decided to adopt method (3) for the subsequent work.

Variation with the fineness of the portion examined. Powdering causes some destruction of individual cells and it is necessary to determine whether the action is selective on either the fibres or the vessel elements. Powder samples Aa1 and AB3 were therefore passed through a series of standard sieves and the fibre/vessel ratio determined on the portions retained by each sieve.

TABLE VI

VARIATION OF THE FIBRE/VESSEL RATIO IN PORTIONS OF THE POWDER OF VARYING FINENESS

Sieve Number				10	22	25	30	36	44	60	85	
Sample	AB.3	—	5.5	5.3	4.0	3.9	4.0	4.2	3.9
Number	Aa.1	8.5	7.3	7.1	7.1	6.4	5.5	5.3	4.8

From these results it is seen that the fibre/vessel ratio decreases with increasing fineness of powder and that the decrease is not large over the 25/44 range. Portions No. 60 and 85 contained a high proportion of broken elements and counting was correspondingly difficult. In view of this and also of that fact that the No. 22/44 portion had been employed previously¹ for vessel index determination, it was decided to employ the latter portion for subsequent work on powders.

Details of the method. About 1.0 g. of the 22/44 portion of the commercial powder was boiled gently with 30 ml. of Schultz's maceration fluid for about 10 minutes or until particles ceased to float on the surface, sufficient potassium chlorate being added meanwhile to maintain a steady evolution of chlorine. The macerated material¹ was then collected on a

THE HISTOLOGY OF BELLADONNA ROOT. PART IV

sintered glass filter, washed with water, transferred to a small test-tube with about 2ml. of water and disintegrated by vigorous shaking. The resulting suspension was then diluted with 3 or 4 volumes of suspending fluid containing a trace of thymol as preservative. Slides were prepared by further dilution such that each scan contained about 20 fibres and the fibre/vessel ratio calculated from observation of the number of vessel elements associated with 300 fibres. In counting, the convention for including broken elements in the count as described above (method 3) was adopted. Counting was done using a 1/6 in. objective and a $\times 6$ eyepiece, and the whole slide covered systematically with the aid of a mechanical stage. Elements intersected by the field of view, but lying more than half within it, were included in the count, otherwise they were ignored.

SUMMARY OF RESULTS

The values of the fibre/vessel ratio for commercial powders are given in Table VII. The ranges are:—

A. Belladonna, 2.30 to 4.55, mean 3.91.

A. acuminata, 6.54 to 12.05, mean 8.66.

These are distinct and the fibre/vessel ratio is thus a good differential character for the powdered drugs. The values for samples Aa1 and Aa2 are slightly higher than those obtained in the preliminary work, which may be accounted for by the fact that they relate to the 22/44 portions, whereas the preliminary work was done on the unsieved powders.

TABLE VII
FIBRE/VESSEL RATIO FOR COMMERCIAL POWDERS—22/44 PORTION

<i>A. Belladonna</i>					<i>A. acuminata</i>						
Code Number					Fibre/vessel ratio	Code Number					Fibre/vessel ratio
AB.1	4.07	Aa.1	6.54
AB.2	4.29	Aa.2	7.0
AB.3	3.91	Aa.3	8.85
AB.4	4.55	*Aa.4	12.05
AB.5	2.35	*Aa.5	8.85
AB.6	2.30						
Mean	3.91	Mean	8.66

* 44/60 portion.

DISCUSSION OF RESULTS

The analysis of variance, Table VIII, for the fibre/vessel ratio determinations on the whole drug shows that the difference between the species is not very significant and hence the differential value of the character when applied to the whole drug is limited. The variance within the species *A. Belladonna* is contributed to almost equally by positional variation within the roots and by differences between them, while in *A. acuminata* root differences are more important. The increase in positional values from the base towards the apex of the roots is fairly uniform and the values at the mid-points approximate to the root means. The critical value for classifying whole roots is 4.27, i.e. half the sum of the specific means, and the expected proportion misclassified on the

basis of a value determined at one position taken at random, as calculated from knowledge of the normal deviate, is 32 per cent. The ranges for the root means are *A. Belladonna* 1.44 to 4.8; *A. acuminata*, 3.03 to 12.01, but little improvement in classification results from their use. However, it may be considered that values less than about 2.0 indicate

TABLE VIII
ANALYSIS OF VARIANCE IN TABLES I AND II

Character	Species	Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Variance Ratio $P=0.01$
Fibre/vessel ratio Table I	<i>A. Belladonna</i>	Roots	9	46.97	5.22	17.7	3.1
		Positions	4	18.65	4.66	15.9	3.9
		Error	36	10.59	0.29		
	<i>A. acuminata</i>	Roots	9	486.34	54.04	31.4	3.1
		Positions	4	52.87	13.22	7.7	3.9
		Error	36	61.91	1.72		
		Species Error	1	150.82	150.82	21.8	7.0
Vessel Index Table II	<i>A. Belladonna</i>	Roots	9	368.51	40.95	63.8	3.1
		Positions	4	9.38	2.35	3.7	3.9
		Error	36	23.10	0.64		
	<i>A. acuminata</i>	Roots	9	2126.68	236.30	99.5	3.1
		Positions	4	92.08	23.02	9.7	3.9
		Error	36	85.52	2.38		
		Species Error	1	3115.10	3115.10	117.1	7.0
			98	2705.27	27.60		

A. Belladonna and greater than about 8.0 *A. acuminata*, intermediate values being inconclusive. In this connection, however, the fact must also be taken into account that the specimens of the whole drug were selected on a basis of diameter and development of xylem and were not taken at random. Reference to the results for commercial powders shows that the mean value of 8.66 for the *A. acuminata* material is considerably higher than that for the whole drug 5.49, indicating that specimens with high fibre/vessel ratios form the greater part of the drug from this species. Thus the misclassification rate of 32 per cent. may be unduly high when applied to specimens taken at random from the commercial drug.

The values of the vessel index for the whole drug also increase from the base towards the apex of the root, but the analysis of variance, Table VIII, shows this effect to be unimportant compared with that due to differences between the specimens. The difference between the species is highly significant, so that the vessel index is valuable for differentiating the whole drug. On the basis of a single determination, the expected misclassification rate is about 15 per cent., the critical value being 9.86 and if the root means are used this proportion is reduced to about 10 per cent. Inspection of the results in Table II does not suggest any close connection between the vessel index and the age of the specimen in *A. Belladonna* although in *A. acuminata* the values for the younger roots numbers 8, 9 and 10 are considerably lower than the mean of the other seven. The ranges for the root means, *A. Belladonna* 0.79 to 8.54, *A. acuminata* 6.99 to 25.38, are not inconsistent with those for another

selection of specimens reported on in Part III of this work, namely. *A. Belladonna* 0.3 to 16.02, mean 6.9 : *A. acuminata* 5.0 to 43.58, mean 19.87. Thus values of less than about 5 may be taken as indicating *A. Belladonna* and greater than 16, *A. acuminata*.

Comparison of the mean values of the vessel index and the fibre/vessel ratio for specimens of the whole drug suggests there is little correlation between them. This is borne out by the low correlation coefficients calculated from the data in Tables I and II, which are :—*A. Belladonna* 0.6, *A. acuminata* —0.15. The two characters may thus be considered as varying independently of one another. Their combination in a linear function yields a discriminant with a critical value for classifying purposes of 14.44 and an expected misclassification rate of 12 per cent. This is a considerable improvement on the figure of 32 per cent. for the fibre/vessel ratio, but is very little superior to that of 15 per cent. when the vessel index is used alone. For this reason, use of the discriminant for other than borderline cases would not be justified.

The variation of the fibre/vessel ratio in the whole drug is not reproduced in the commercial powder owing to the thorough mixing of the elements on which it is based. Thus the values for commercial powders vary within narrow limits and, since the ranges for the two species are distinct, the fibre/vessel ratio provides a valuable character for their differentiation.

SUMMARY AND CONCLUSIONS

1. The term "fibre/vessel ratio" is adopted to signify the number of fibres (including fibre-tracheids exhibiting elongated simple pits) associated with one vessel element.

2. Consistent results are obtained when the fibre/vessel ratio is calculated from the number of vessel elements associated with not less than 200 fibres.

3. The variation in the whole drug of the fibre/vessel ratio and of the vessel index 135μ is investigated and their value as differential characters assessed.

4. Neither character is completely successful in differentiating the whole drug, but some improvement results by combining them in a linear function.

5. A method of determining the fibre/vessel ratio on the powdered drug is described.

6. The fibre/vessel ratio of commercial belladonna root powders decreases with the fineness of the portion examined, but is reasonably constant for the portion retained between a No. 22 and No. 44 sieve.

7. Fibre/vessel ratio values for commercial powders provide a means of differentiating the species from which they were prepared.

8. The ranges for the commercial powders examined are :—*A. Belladonna* 2.30 to 4.55 ; *A. acuminata* 6.54 to 12.05.

REFERENCES

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2. Mather, *Statistical Analysis in Biology*, Methuen, 1943.
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action of khellin and of the glycoside (which he respectively called visammin and khellinin) and found that khellin increases the flow of urine and causes a relaxation of the visceral plain muscles. The interest in the crystalline constituents of *Ammi Visnaga* has been recently revived as the result of the demonstration by Anrep, Barsoum, Kenawy and Misrahy¹⁰ that khellin causes a conspicuous dilatation of the coronary blood vessels without much affecting the systemic circulation. The interest in the substance was further stimulated by the promising results of the clinical observations upon the effect of khellin in the anginal syndrome (Kenawy and Barsoum¹¹, Anrep and *et al.*¹²² Ayyad¹³).

Salama¹⁴ showed in animals and man that none of the crystalline principles of *Ammi Visnaga* exerts a diuretic action, the increased formation of urine being entirely due to the fluid taken in the form of the decoction.

Samaan^{15,16} ascribes the coronary vasodilator action of *Ammi Visnaga* not to khellin, but to the glycoside, which, according to him, causes in concentrations of 1 in 25,000 and even 300,000 a conspicuous increase of the coronary outflow of the isolated perfused rabbit's heart. In experiments in which an artificial spasm of the coronary blood vessels was induced by barium chloride, administration of the glycoside was stated to cause sometimes as much as a twelve-fold increase of the coronary outflow. On the other hand, according to Bagouri¹⁷, the glycoside, even when used in high concentrations, exerts no action on the coronary vessels of the perfused heart, the coronary vasodilatation being entirely due to khellin.

It follows from the above that further research is required before a proper assessment of the pharmacological potency of the different crystalline principles of *Ammi Visnaga* can be made. We, therefore, undertook to investigate the comparative action of khellin, of visnagin, of the glycoside and of their derivatives by quantitative methods.

METHODS OF PREPARATION

The three natural crystalline substances of *Ammi Visnaga*, khellin, khellol glycoside and visnagin were prepared according to the method devised by Späth and Gruber. The substances were repeatedly crystallised from methyl alcohol and other solvents until their respective melting-points reached the maximum values given by the Austrian observers and did not change by further recrystallisation. The purity of the final products was controlled in the Pharmacognosy Laboratory and in the Faculty of Science of this University. The fission products, khellinon, visnaginon and khellol were obtained by the action of acid and alkali as recommended by Späth and Gruber. The purification of these substances presents no difficulty since they easily crystallise from methyl alcohol and give sharp melting-points, 100°C. for khellinon, 110°C. for visnaginon and 179°C. for khellol.

THE COLORIMETRIC AND THE BIOLOGICAL ASSAY OF THE CRYSTALLINE PRINCIPLES OF *Ammi Visnaga*

Colorimetric assay:—The moderately stable pink coloration which khellin gives, as discovered by Fahmy and El-Keiy⁴, in contact with solid sodium hydroxide, served as the basis for the colorimetric assay of khellin and of visnagin. For quantitative work we used a saturated solution of potassium hydroxide and a 0.5 millimolar standard solution of khellin in water (0.13 mg. of khellin per ml.). Addition of 0.1 ml. of the khellin solution to 1.0 ml. of

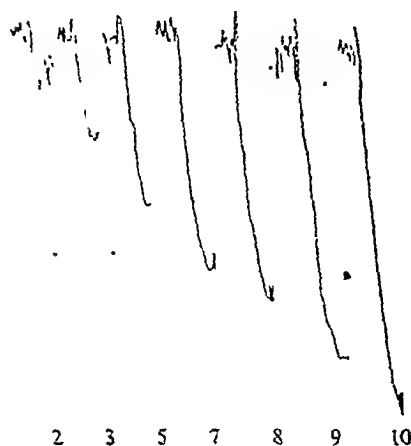


FIG. 1 Response of the rectal caecum of the fowl to gradually increasing doses of khellin. The amounts administered are shown in μ g.

used the rectal caecum of the fowl bathed in Tyrode's solution in a bath 5 ml. in capacity. We find that the rectal caecum can be satisfactorily

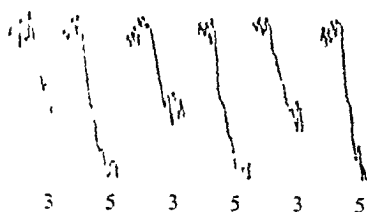


FIG. 2 Regular response of the rectal caecum to alternate doses of 3.0 and 5.0 μ g. of khellin

saturated potassium hydroxide solution gives an intensity of colour suitable for work with a sensitive colorimeter of the ordinary type or with a photoelectric colorimeter. As has already been shown by Fahmy, this method is less suitable for the estimation of the glycoside, which, in presence of strong alkali, gives a very unstable cherry-red colour. The products of acid and alkaline hydrolysis of the three natural substances give no pink coloration in presence of strong alkali.

Biological assay:—The biological method of assay used in this work was originally devised by Barsoum and Gaddum¹⁸ for the estimation of adenosine. As a test object they used the rectal caecum of the fowl suspended in Tyrode's solution in a bath 5 ml. in capacity. We find that the rectal caecum can be satisfactorily used also for the comparative assay of the active principles of *Ammi Visnaga*. With sensitive preparations it is possible to make the assay with an accuracy of about 10 per cent. Figure 1 shows the reaction of the rectal caecum to gradually increasing doses of khellin which, for this purpose, was dissolved in Tyrode's solution. An average preparation of the rectal caecum is sensitive to about 2 μ g. of khellin, i.e., a concentration of khellin in the

bath of 2.5×10^{-7} . Figure 2 shows that repeated administration of the same dose of khellin gives the same degree of relaxation of the rectal caecum, and Figure 3 serves as an example of a comparative assay of visnagin and of khellin showing that visnagin is about 30 per cent. less active than khellin.

Table I gives the results obtained with the three natural substances and with the products of their hydrolysis. The results of the colorimetric and of the biological assays given in Table I are means of not less than 50 separate estimations of the 3 natural substances and of their derivatives. Usually only 2 or 3 substances were assayed on one rectal cæcum. Before administration, the solutions were warmed to the temperature of the bath containing the cæcum. The doses of the substances were such as to cause 30 to 50 per cent. of the maximal relaxation of the intestinal muscle, which is the range of maximal discrimination of the preparation.

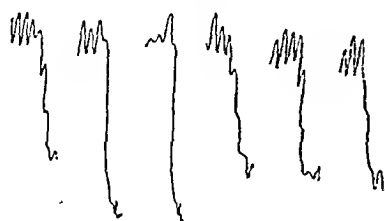


FIG. 3. Comparative assay of khellin and of visnagin on the rectal cæcum of the fowl. The amounts of the two substances administered are given in μg .

of the intestinal muscle, which is the range of maximal discrimination of the preparation.

TABLE I

COLORIMETRIC AND BIOLOGICAL ASSAYS OF KHELLIN, OF VISNAGIN, OF KHELLOL-GLYCOSIDE AND OF THE PRODUCTS OF THEIR HYDROLYSIS. ALL THE SUBSTANCES WERE PREPARED IN 0.5 MILLIMOLAR SOLUTIONS AND KHELLIN WAS TAKEN AS THE STANDARD FOR THE COMPARISON

EQUIVALENT DOSES IN μg .

		Khellin m.pt. 154°C	Visnagin m.pt. 144°C	Glycoside m.pt. 175°C	Khellinon m.pt. 100°C	Visnaginon m.pt. 110°C	Khellol m.pt. 179°C
Colorimetric assay	...	10	14.5	not tested	gives no colour reaction	gives no colour reaction	not tested
Standard deviation	...	—	0.5	—	—	—	—
Biological assay	...	10	15	over 300	30	50	40
Standard deviation	...	—	0.6	—	1.1	2.2	—
Percentage of activity in relation to that of Khellin							
		100	66	traces	33	20	25

Table I reveals the following points of interest:

- (1) Colorimetric and biological assays show that the monomethoxy derivative, visnagin, is about 30 per cent. less active than the dimethoxy derivative, khellin. The agreement between the colorimetric and the biological assays is extremely satisfactory.
- (2) Khellinon, the alkali-split product of khellin, loses its colour reaction, but partially retains its power to relax the smooth muscle. Biologically, khellinon is about one third as active as khellin.
- (3) The biological action of the glycoside, as tested on the rectal cæcum, is not less than 30 times weaker than that of khellin. In fact it is questionable whether it has any action at all.
- (4) Khellol, the product of acid hydrolysis of the glycoside, shows a considerably greater activity than the mother substance. Apparently the substitution of the glycoside radical by the hydroxyl group partially unmask some of the latent activity of the rest of the molecule.

THE PHARMACOLOGICAL ACTIONS OF *AMMI VISNAGA* LINN.

- (5). Khellol is a monomethoxy derivative which differs from visnagin by containing a hydroxyl group. This difference is sufficient to cause a conspicuous diminution of the activity of khellol as compared with that of visnagin.
- (6) Visnaginon, similarly to khellinon, gives no colour reaction. Its biological action is about 30 per cent. weaker than that of khellinon.

The action of khellin on the rectal cæcum is considerably weaker than that of adrenaline, 4 μ g. of khellin being approximately equivalent in action to 0.01 μ g. of adrenaline chloride. Khellin is, however, about 12 times more effective than aminophylline. The rabbit's uterus is also relaxed by khellin, especially when it has previously been contracted by administration of adrenaline, showing that khellin acts directly on the plain muscle and not on the sympathetic nerve endings.

THE COMPARATIVE ACTION OF KHELLIN, OF VISNAGIN AND OF THE KHELLOL GLYCOSIDE ON THE CORONARY CIRCULATION

At the time when Anrep and *et al*¹⁰ made their observations upon the coronary vasodilator action of khellin in the heart lung preparation, the existence of the related monomethoxy compound, visnagin, was not yet known. So far, visnagin does not present much interest from the practical point of view, since it occurs in the fruit of *Ammi Visnaga* in very small amounts. However, in the future it might possibly be prepared synthetically. From the theoretical point of view it presents a greater interest because a comparison between visnagin, khellin and the glycoside might throw a light on the relation between the action of these substances and their molecular structure. The comparison of the action of khellin and of the glycoside on the one hand and of khellin and of visnagin on the other, was made on the standard heart-lung preparation on dogs by collecting the blood through a coronary sinus cannula. A few typical experiments, selected from amongst many others, are sufficient to illustrate the action of these substances.

Experiment 1. Heart-lung preparation: systemic output 650 ml./minute; aortic blood pressure 120 mm. Hg. For about 20 minutes the coronary outflow remained constant at 42 to 44 ml./minute. After a gradual administration of 40 mg. of the glycoside the coronary blood flow remained unchanged. Administration of 5 mg. of khellin increased it to 59 ml./minute; after another dose of 5 mg. of khellin the flow increased to 90 to 95 ml./minute. The total amount of blood in circulation was about 800 ml.

Experiment 2. Heart-lung preparation; output 500 ml./minute; aortic blood pressure 120 mm. Hg. The outflow of blood from the coronary sinus remained constant for over 15 minutes at 58 to 61 ml./minute. 4 doses of the glycoside, 20 mg. each, were administered at intervals of a few minutes; 80 mg. in all. The coronary blood flow remained unchanged although the drug was allowed to circulate for several minutes. Administration of 10 mg. of khellin rapidly increased the coronary blood flow to 120 ml./minute. The total amount of blood in circulation was about 700 ml.

Experiment 3. Demonstrated to the Cairo Clinical Society. Heart-lung preparation output 450 ml./minute, arterial blood pressure 130 mm. Hg. The outflow from the coronary sinus was 51 ml./minute. On administration of 45 mg. of the glycoside the coronary outflow remained the same. After administration of 15 mg. of khellin it increased to 250 ml./minute. The amount of blood in circulation was about 500 ml.

The action of khellin was extremely prolonged, the coronary blood flow remaining increased to the end of an experiment. In this, the effect of khellin greatly differs from that of amyl nitrite.

In some of the experiments the action of khellin on the coronary blood flow was more and in others less conspicuous than in the above examples. As regards the glycoside, no coronary vasodilator action could be demonstrated in the heart-lung preparation, even though its concentration was increased to 100 μ g./ml.

We were also able to confirm the observation of Bagouri¹¹, who found that systemic blood vessels are much less sensitive to the vasodilator action of khellin than coronary blood vessels, and that the glycoside caused no coronary dilatation in the perfused rabbit's heart.

The glycoside, since it has no action on the coronary blood vessels, could be administered together with khellin in the same heart-lung preparation. This is not possible when comparing the action of khellin with that of visnagin. Both are coronary vasodilators, the action of which persists for a very long time. The action of the two drugs was, therefore, studied on two separate preparations which were made to work in, as nearly as possible, the same experimental conditions. The type and the weight of the dogs used for the two preparations were the same; the arterial blood pressure, temperature and the output of the heart were respectively maintained at the same levels, and the two hearts usually did not differ in weight by more than 5 g. In spite of these precautions, the individual variations of the coronary sinus outflow were too large to permit of a definite conclusion as regards the relative action of the two drugs. The observations made on the rectal cæcum would suggest that visnagin might possibly be a somewhat weaker coronary vasodilator than khellin. Observations made on two separate heart-lung preparations are not, however, sufficiently accurate to justify this conclusion. As compared with aminophylline the action of khellin on the coronary circulation in the heart-lung preparation is 4 to 6 times stronger.

OBSERVATIONS ON THE ALIMENTARY TRACT *in situ*

The experiments were made on dogs of 7 to 9 kg. weight, anæsthetised with chloralose, sodium luminal or nembutal. The abdomen was opened by a median incision and a loop of the jejunum, about 30 to 40 cm. in length, was tied off between two ligatures. A wide cannula was inserted into each end of the loop. The two cannulae were then connected to a separating funnel the top of which was joined to a volume recorder. The funnel, filled with saline solution, was kept at

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a suitable height, sufficient to fill but not to distend the intestinal loop. The contractions of the loop were recorded on a drum. Intravenous injection of khellin in doses of 5 to 10 mg. caused a rapid and conspicuous relaxation of the intestinal loop, the rhythmic movements being

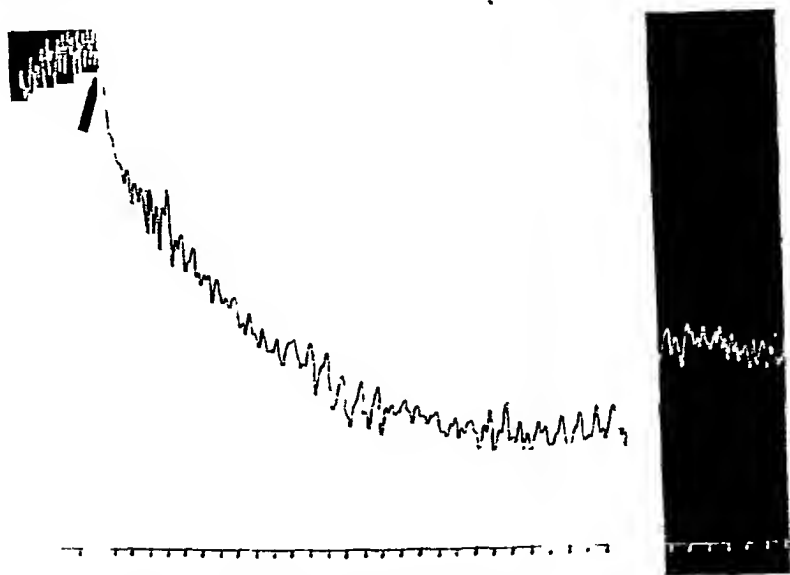


FIG. 4. Dog 10 kg. Left, effect of intravenous injection of 10 mg. of khellin on the intestinal movements recorded as described in the test. Right, 20 minutes later. Time in 10-second intervals.

reduced in rate and in strength. The relaxation of the intestine following administration of khellin was extremely prolonged, the recovery being slow and usually incomplete even after 1 to 2 hours. Figure 4 shows the effect of administration of khellin on the intestinal movements in the whole animal. Khellol glycoside even in greater doses caused no relaxation of the intestinal loop and no diminution of the rhythmic contractions (Fig. 5). In some experiments injections of the glycoside were followed by an increase of the intestinal tone.

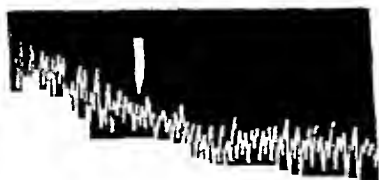


FIG 5. Dog 9.5 kg. Effect of injection of 20 mg. of khellol glycoside on the intestinal movements. Times in 10-second intervals. The intestine was slowly relaxing before the injection

OBSERVATIONS ON THE BRONCHIAL MUSCLES

Samaan" showed that khellin causes a relaxation of bronchial muscle, but did not study the effect by quantitative methods. Our ex-

periments were performed on perfused lungs of the guinea-pig, using the method of Sollmann and Oettingen¹⁹. The lungs were suspended in an air thermostat at a temperature of 38° to 40° C., the air being kept saturated with water. A cannula was introduced into the trachea, and the lungs were perfused with warm oxygenated Ringer-Locke solution in the same manner as the isolated heart. In order to allow an outlet, the surface of the lung was scarified in several places, the fluid being collected through a wide funnel in a graduated cylinder. All the measurements were made without opening the thermostat so as to avoid cooling the lungs. The results of some of the experiments are given in Table II.

TABLE II
ACTION OF KHELLIN ON THE BRONCHI OF THE PERFUSED LUNGS OF THE GUINEA-PIG

Initial flow in ml./minute	Concentration of khellin in mg./l.	Maximum flow in ml./minute	Percentage increase
5.0 to 5.4	10	42.0 to 43.0	700
4.4 to 4.8	10	39.0 to 39.6	750
2.5 to 2.6	6	11.6 to 12.0	350
3.3 to 3.5	4	9.8 to 10.2	195
3.5 to 3.7	2	5.2 to 5.4	47

Other similar experiments gave approximately the same results. The minimum effective concentration of khellin causing a 50 per cent. increase in the outflow was about 2 to 3 $\mu\text{g./ml.}$ After replacing the khellin solution with Ringer-Locke solution the outflow of the fluid from the bronchial tree usually returned to its original volume in the course of 4 to 5 minutes. The effect of khellin is strong enough to antagonise considerable concentrations of histamine. When the lungs are perfused with 10 mg./l. of khellin, injections of 5 to 10 $\mu\text{g.}$ of histamine diphosphate have only a negligible effect. In absence of khellin these doses cause a conspicuous broncho-constriction. The glycoside even in high concentrations causes no relaxation of the bronchi. As a broncho-dilator khellin is 4 to 6 times more effective than aminophylline.

As a result of the above observations administration of khellin was tried in a large number of patients suffering from bronchial asthma. With a few exceptions, presented by subjects with advanced emphysema or fibrosis of the lungs, khellin was extremely beneficial. The asthmatic attacks were cut short, the vital capacity of the subjects increased and the feeling of respiratory distress disappeared. On continuous administration of khellin the attacks either disappeared or became less frequent and less severe. The treatment of bronchial asthma with khellin will form the subject of another communication.

ESTIMATION AND DISTRIBUTION OF KHELLIN IN BLOOD AND TISSUES

The estimation of khellin in blood and tissues can be made by the colorimetric method or by the biological method on the rectal caecum. The following procedure was adopted for the preparation of the final extracts suitable for quantitative estimations. Ten ml. of blood was

added to 100 ml. of alcohol; after filtration the precipitate was washed with three quantities of alcohol, each of 10 ml. The washings and the filtrate were mixed and evaporated on a water-bath, under reduced pressure, to a volume of about 5 ml.; 50 to 100 ml. of water was then added and the solution was treated in a separating funnel with 3 quantities of chloroform, each of 10 ml., to extract the khellin. The chloroform solution was evaporated to complete dryness and the residue was dissolved in exactly 1 or 2 ml. of distilled water, for the colorimetric test, or, of Tyrode's fluid, for the biological assay. This method presents the advantage that the khellin content of any reasonable quantity of blood or of other biological fluids can be concentrated in the 1 or 2 ml. of the final extract. The colorimetric or the biological assay was made against a standard 0.5 millimolar solution of khellin. The recovery of khellin by the above method is 95 to 100 per cent. When the method is used for tissues, a piece of an organ is weighed, ground with silver sand, treated with alcohol and extracted with chloroform as described for blood. Control observations showed that the extraction of khellin from tissues is somewhat less complete, ranging between 85 and 95 per cent. Extraction of khellin from fat is less satisfactory, khellin being highly soluble in lipoids. The accuracy of the colorimetric and of the biological method is the same.

Distribution of khellin in blood.—Khellin added to defibrinated blood or to blood rendered incoagulable by addition of heparin is taken up by the serum or plasma and by the red blood corpuscles. With concentrations varying between 1 and 200 $\mu\text{g./ml.}$ the plasma or serum contained about 10 to 20 per cent. more khellin than the red blood corpuscles. This proportion is not changed by allowing the blood to stand for several hours before it is centrifuged. It is well known that the red blood cells are able to take up a large number of organic substances, some of which are easily given off, while others become fixed and, therefore, probably biologically inactive. Glucose, for example, belongs to the first group of substances and histamine to the second. We find that khellin is rapidly given off by the corpuscles when these are exposed to serum or Tyrode's solution containing no khellin. It is, therefore, obvious that the khellin of the red blood corpuscles is not pharmacologically wasted. It should be looked upon as a store which is readily released to the surrounding plasma.

After intravenous or intramuscular administration of khellin the drug at first appears in the blood in a high concentration. Within a few minutes the khellin concentration begins to diminish, and in about 20 to 30 minutes it reaches a steady level which is maintained for several hours. The rapid diminution of the khellin concentration in the circulating blood is not due to excretion by the kidneys or to destruction by the tissues, but to a gradual and more or less uniform distribution of the drug amongst all the organs of the body.

The drug remains in the circulation for an extremely long time. In dogs after injections of 10 to 20 mg./kg. khellin could be detected in

the blood as long as 36 hours later; 24 hour-samples of urine collected after the injection contain only traces of khellin. The conclusion must be, therefore, made that the khellin is not eliminated by the kidneys in an unchanged form.

Repeated administrations of khellin lead to its accumulation in the blood and tissues. Animals injected with doses of 10 mg./kg. had a concentration of 4 $\mu\text{g./ml.}$ of blood, 24 hours after the first injection and 12 $\mu\text{g./ml.}$ 24 hours after the ninth injection. A similar accumulation of the drug can be also demonstrated in man. For example, in subjects who received one injection of 200 mg. of khellin per day its concentration in the blood, 30 minutes after the first injection, was 4 to 5 $\mu\text{g./ml.}$ and after the 5th injection, 12 to 17 $\mu\text{g./ml.}$

In order to study the distribution of khellin in the tissues the drug was injected intramuscularly in doses of 20 to 40 mg./kg., the administration of such large doses being necessary since only small samples of tissues could be used to obtain a perfect extraction. The dogs were killed at different intervals of time after the injections and their tissues analysed. In one set of experiments the first animal was killed 1 hour after the injection, the second 24 hours, and the third 36 hours later. The concentration of khellin in the blood of these animals was 30, 16 and 5 $\mu\text{g./ml.}$ respectively. The concentration in the liver, muscle, brain, kidney and the mesentery varied between 20 and 40 $\mu\text{g./g.}$ 1 hour after the injections, between 7 and 15 $\mu\text{g./g.}$ 24 hours and between 2 and 5 $\mu\text{g./g.}$ 36 hours later. It follows that khellin is rapidly distributed over the whole body. The concentration in the liver was always somewhat higher than in the other tissues. The figures obtained for the brain were the lowest, which is probably due to the difficulty of extraction of khellin from lipoid-containing tissues. The disappearance of khellin from the tissues is extremely slow and is not related to any particular organ.

ABSORPTION OF KHELLIN AND OF THE KHELLOL GLYCOSIDE FROM THE ALIMENTARY TRACT

Khellin is absorbed from the stomach, from the small intestine and from the large intestine. The absorption from the stomach and from the small intestine was studied in anæsthetised dogs after complete separation of the pylorus from the duodenum. Khellin solutions were injected directly into the stomach or into the duodenum. In some of the experiments khellin was injected into a separated loop of the small intestine. Absorption from the large intestine was studied only in man. The blood samples were collected after the respective injections and assayed for khellin in the usual manner. The absorption from the alimentary tract is not followed by a temporary large increase of the khellin concentration in the blood as is the case with intramuscular absorption. Oral administration is, therefore, suitable for the maintenance of a high concentration of khellin in the blood, while intramuscular

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administration is more suitable when it is desired to raise its concentration in a short time.

In man, after oral administration of 300 mg. the maximum concentration of 5 to 6 $\mu\text{g./ml.}$ of blood was reached in 40 to 60 minutes. After rectal administration of 500 mg. dissolved in 50 ml. of alcohol (20 per cent.), the maximum concentration of 6 to 8 $\mu\text{g./ml.}$ was reached in about 2 hours. It can be seen that the rate of absorption of khellin from the large intestine is not inferior to that from the rest of the digestive tract.

No evidence could be found to show that the khellol glycoside is converted in the body to active khellin. After oral or intramuscular administration of 4 to 5 g. of the glycoside, in non-anæsthetised dogs, no khellin could be detected in their circulating blood. Neither is there any evidence showing that the glycoside is absorbed from the intestinal tract. In dogs, anæsthetised with chloralose, a loop of the small intestine, about 50 cm. long, was tied off and its two ends were provided with cannulæ. After thoroughly washing the inside of the loop, 70 ml. of a solution containing 1 mg./ml. of khellin or of the glycoside in alcohol (20 per cent.) was injected into the loop, which was then closed and returned to the abdominal cavity. After 30 to 90 minutes the loop was emptied into a measuring cylinder and washed 2 or 3 times with alcohol (20 per cent.) to remove all traces of the injected substance. The amount of khellin or of the glycoside which escaped absorption was then determined. The khellin was determined as described before for blood and the glycoside, by measuring the reducing power of the intestinal content before and after hydrolysis in acid following a preliminary precipitation of the protein matter with alcohol (95 per cent.). The results of 4 experiments are given in Table III.

TABLE III

ABSORPTION OF KHELLIN AND OF KHELLOL GLYCOSIDE FROM THE SMALL INTESTINE
AMOUNT OF FLUID, ALCOHOL (20 PER CENT.), INJECTED INTO THE LOOP WAS IN EACH
CASE 70 ML. CONTAINING 70 MG. OF KHELLIN OR OF THE GLYCOSIDE

Substance injected	Duration of absorption in minutes	Amount of fluid not absorbed ml.	Amount of substance not absorbed mg.
Khellin	30	43	14
Khellin	30	41	17
Glycoside	30	48	68
Glycoside ..	90	26	72

CONCLUSIONS

1. A biological method of assay and a modification of the colorimetric method of assay of the active crystalline principles of *Ammi Visnaga* are described.
2. A comparative colorimetric and biological assay of khellin, of the khellol glycoside and of visnagin showed that the glycoside is biologically almost inactive and that the activity of visnagin is about one-third less than that of khellin.

3. The glycoside has no detectable action on the coronary circulation.
4. No difference could be detected between the coronary vasodilator action of khellin and of visnagin.
5. Kellinon and visnaginon, the products of alkali hydrolysis of khellin and visnagin respectively, give no colour reaction, but still exert some biological activity. The action of khellinon is about one-third that of khellin, and the action of visnaginon about one-third that of visnagin.
6. Khellol, the product of acid hydrolysis of the glycoside, has a definite biological action which is much stronger than that of the glycoside and is about 25 per cent. of that of khellin.
7. Khellin causes a conspicuous relaxation of the bronchi in the isolated lungs and a diminution of the intestinal tone in the whole animal. The khellol glycoside is in these respect inactive.
8. Khellin is rapidly absorbed from the small intestine, from the stomach and from the large intestine.
9. Intramuscular injections of khellin are followed by a rapid and conspicuous increase of its concentration in the circulating blood, which after some time gradually diminishes and finally becomes stabilised at an approximately uniform level. Oral administration is not followed by such a temporary large increase; the concentration of khellin increases gradually and reaches a stable level in about 30 minutes.
10. Khellin is not excreted in the urine in an unchanged form and it disappears from the blood and tissues at a very slow rate.
11. In man, oral or intramuscular administration of a single dose of 100 to 200 mg. of khellin raises its concentration, in the blood to above the minimal effective concentration, which has been shown to cause a coronary vasodilatation and relaxation of the bronchi. Due to the slow destruction of the drug, repeated administration leads to its accumulation in the body.
12. No evidence could be found that the khellol glycoside is converted in the body to khellin or that it can be absorbed from the intestine in an unchanged form.

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THE CORONARY VASODILATOR ACTION OF THE CRYSTALLINE PRINCIPLES OF *AMMI VISNAGA* LINN.

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THE TWO MAIN CRYSTALLINE PRINCIPLES of the fruit of *Animi Visnaga*, khellin and khellol glycoside, have been extracted by Mustafa,¹ Malosse,² Fantl and Salem,³ Fahmy and El-Keiy,⁴ and Samaan.⁵ The chemical structure of the two compounds was determined by Späth and Gruber.^{6,7} Samaan claims that khellin (named by him visammin) causes a relaxation of visceral plain muscle, while the glycoside (named by him khellinin) conspicuously dilates the coronary blood vessels of the perfused rabbit's heart.^{8,9} The latter observation stands in direct contradiction with that of Anrep, Barsoum, Kenawy and Riad,¹⁰ who find, in the heart-lung preparation on dogs, that the glycoside has no effect on the coronary circulation and that the relaxation of plain muscle, as well as the coronary vasodilation are both due to khellin. The question arises whether possibly both substances, khellin as well as the glycoside, have a coronary vasodilator action, the difference in the results being due to the difference in the species of animals used or to the fact that Samaan worked on isolated hearts perfused with Ringer-Locke's solution, while the experiments of Anrep *et al.* were performed on hearts supplied with blood.

The object of the experiments described in this communication was to compare the action of khellin and of the glycoside on the coronary circulation in the isolated perfused rabbit's heart. I should like to thank Prof. Fahmy and Dr. Haddad, of the Pharmacognosy Department, for the supply of the two substances. Some of the experiments were made with the khellin and the glycoside prepared in the Physiological Laboratory or obtained from pharmaceutical firms. These samples will not be described separately, since no difference in their action could be detected.

COMPARATIVE ACTION OF KHELLIN AND OF KHELLOL GLYCOSIDE ON THE CORONARY CIRCULATION OF THE ISOLATED PERFUSED RABBIT'S HEART

The administration of the different substances was made by changing the fluid perfusing the isolated heart from oxygenated Ringer-Locke's solution to the same solution in which the one or the other of the two substances had been dissolved. A modified form of Langendorff's method was used for the perfusion. The coronary outflow was recorded by collecting the fluid in a measuring cylinder at intervals of 30 seconds. The perfusion pressure was kept at 100 to 120 mm. Hg. A few examples representing the average results obtained are given in Table I.

Khellin in such a large concentration as used in the above experiment, caused a diminution in the amplitude of the heart beat, which was in

EXPERIMENTS WITH KHELLIN

TABLE I

PERFUSION OF THE ISOLATED RABBIT'S HEART. KHELLIN CONCENTRATION 40 mg./l.

Perfusion Fluid	Coronary outflow in ml./minute measured at intervals of 30 seconds
1—Ringer Locke's solution ...	9, 9, 8.6, 9, 9, 9.2, 9, 9
2— " " with Khellin ...	12, 19, 24, 28, 35, 36, 38, 37.5, 38, 39
3— " " solution ...	35, 33, 28, 24, 22, 17, 16.5, 11, 11, 9, 9.5, 9, 9, 9.5, 9
4— " " with Khellin ...	12, 16, 19, 22, 28, 36, 38, 40, 39, 40, 39
5—7 minutes later ...	35, 36, 38, 35, 37, etc.
6—Ringer Locke's solution ...	33, 30, 26, 22, 20, 18, 14, 13, 10, 8.5, 8, 8

most cases of a temporary nature. It is unnecessary to give a detailed description of each experiment, instead, a summary, showing the initial coronary outflow and the maximum increase obtained with different concentrations of khellin, is given in Table II.

TABLE II

INITIAL CORONARY OUTFLOW AND THE MAXIMUM INCREASE OBTAINED WITH DIFFERENT CONCENTRATIONS OF KHELLIN

Average initial coronary outflow in ml./minute	Concentration of khellin mg./l.	Maximal coronary outflow during perfusion with khellin ml./minute	Increase per cent.
9.0	40.0	39.0	333
9.0	40.0	40.0	334
4.5	10.0	12.0	167
4.2	10.0	10.8	157
10.2	4.0	15.5	52
9.2	4.0	14.5	58

Concentration of 2 to 10 mg./l. caused no detectable effect on the heart beat. As regards the coronary circulation, the minimum effective concentration of khellin, for the rabbit's heart perfused with Ringer's solution, is somewhat below 2 mg./l., i.e., about double that given by Anrep, Barsoum, Kenawy and Misrahy¹¹ for the heart lung preparation.

In several experiments, solutions of 1:40,000 or 1:50,000 of barium chloride were used to induce an artificial spasm of the coronary blood vessels before perfusion with khellin. No special advantage was, however, gained by this procedure. In the presence of barium chloride, larger concentrations of khellin had to be used to cause an appreciable increase of the ordinary outflow. The coronary vasodilator action of khellin can be demonstrated on the normally beating heart as well as on the fibrillating heart.

EXPERIMENTS WITH KHELLOL GLYCOSIDE

The observations with the glycoside were carried out with the same technique as those with khellin. The results obtained in some of the typical experiments are summarised in Table III.

Every observation was made on a different heart. In all the above experiments, administration of khellin caused the usual coronary dilata-

THE CORONARY VASODILATOR ACTION OF AMMI VISNAGA LINN.

TABLE III
EFFECT OF KHELLOL GLYCOSIDE ON CORONARY OUTFLOW

Average initial coronary outflow ml./minute	Concentration of the glycoside mg./l.	Average coronary outflow during perfusion with the glycoside ml./minute
11.0	4.0	10.5
6.5	4.0	6.8
10.8	20.0	11.2
8.8	20.0	8.8
12.0	20.0	11.5
7.4	40.0	5.2
5.8	40.0	4.4
4.7	100.0	4.5
5.9	100.0	5.2

tion. Attempts to demonstrate the coronary vasodilator action of the glycoside after inducing a coronary spasm by means of barium chloride were unsuccessful. In fact, in many experiments, administration of large doses of the glycoside caused some diminution of the coronary outflow, but never an increase. The diminished outflow does not necessarily indicate a vaso-constriction, since it may be accounted for by the mechanical effects accompanying the slight increase in the strength of the heart beat, which is usually observed on administration of large doses of the glycoside.

COMPARATIVE ACTION OF KHELLIN AND OF THE KHELLOL GLYCOSIDE ON THE ISOLATED CORONARY AND SYSTEMIC ARTERIES

The study of the action of drugs on isolated arterial rings was first made by Langendorff. A detailed description of the results obtained by this method was given by Cruickshank and Subba Raw.¹² These authors observed some fundamental difference in the reaction of coronary and systemic arterial rings to changes in temperature and to different drugs. Kountz,¹³ working in this laboratory, confirmed the results of the previous workers and applied the method to human arterial rings. The same method was used for the study of the action of khellin and of the glycoside.

Intramuscular branches of the coronary arteries of the water buffalo were dissected and several rings, about 2 mm. thick, were joined together by means of silver wire, 3-4 rings in a chain. The rings were mounted in a 50-ml. bath containing oxygenated Ringer-Locke's solution at 37° C. The contractions of the rings were recorded by a light lever allowing a magnification of about 30 to 40 times. The drugs were administered directly into the bath.

Khellin, in doses of 0.5 mg., caused a definite relaxation of the rings. After replacing the khellin-containing solution with fresh Ringer-Locke's fluid the rings showed a partial recovery, never, however, completely regaining their original tone. Both the relaxation and the recovery were very slow. The difference between the action of khellin and of the glycoside was quite obvious. The latter caused no relaxation of the coronary rings, even though the doses were increased to about 10 times above those of khellin.

As regards the action of khellin upon rings of systemic arteries, I found that doses up to 2 mg. caused no relaxation. Evidently the systemic arteries are much less sensitive to the drug than the coronary arteries.

In order to gain further knowledge about the action of khellin and of the glycoside on systemic blood vessels, I made use of Pissemsky's method¹⁴ of the perfused rabbit's ear. This method presents the advantage that the perfusing fluid need not be oxygenated or warmed.

Samaan found that the flow of fluid through the perfused toad may be as much as doubled by khellin in a concentration of 1:5,000. Such high concentrations present no therapeutic interest. My own observations confirm the statement of Anrep, Barsoum, Kenawy and Misrahy,¹¹ that khellin in concentrations which cause a conspicuous dilatation of coronary blood vessels has no effect on the systemic blood vessels, the latter being less sensitive. Khellin as well as the khellol glycoside, in concentrations up to 40 mg./l., caused no increase in the flow of the Ringer-Locke's solution through the perfused rabbit's ear.

CONCLUSION

1. Khellin causes a conspicuous increase in the coronary outflow in the isolated perfused rabbit's heart, the minimum effective concentration being about 2 mg./l.
2. The khellol glycoside causes no increase in the coronary outflow, even when administered in concentrations as high as 100 mg./l.
3. Isolated coronary rings are relaxed by khellin and are not affected by the glycoside.
4. The systemic blood vessels are considerably less sensitive to khellin than the coronary blood vessels.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS .

CHEMISTRY

ALKALOIDS

Fluosilicates of the Alkaloids. Janot and Chaigneau. (*C. R. Acad. Sci., Paris*, 1948, **227**, 982.) Knowledge of the fluosilicates of the alkaloids and related substances has been extended to the analysis and optical rotation of 13 alkaloidal fluosilicates. The fluosilicic acid was determined as the potassium salt in alcohol (50 per cent.); in the case of the morphine compound as the fluorochloride of lead. The alkaloid was liberated with ammonia and extracted with ether, chloroform or amyl alcohol. The fluosilicates crystallised easily in prismatic needles or plates; the quinine and quinidine compounds showed a blue, those of morphine and corynanthine a green, fluorescence. The general formula was $\text{SiF}_5\text{H}_2(\text{alkaloid})_2\text{XH}_2\text{O}$, except the compounds of morphine and codeine, both of which were anhydrous. H. F.

ANALYTICAL

Colorimetric Determination of Copper with Carbon Disulphide and Diethanolamine. W. C. Woelfel. (*Anal. Chem.*, 1948, **20**, 722.) Use has been made of the reaction of the bis-(2-hydroxy-ethyl)-dithiocarbamate of diethanolamine with the cupric ion forming a brownish yellow salt soluble in water, as the basis of a colorimetric method for the determination of copper. The reagent is prepared by mixing solutions of carbon disulphide and diethanolamine, both in methyl alcohol. Several advantages are claimed over the ordinary diethyldithiocarbamate procedure in that the solubility of the coloured copper salt eliminates the need for a stabilising colloid or for extraction with an organic solvent. Of the metals whose compounds are soluble under the conditions used, bismuth, chromium, cobalt, iron, mercury, nickel, silver, and uranium interfere seriously. Procedures are described for eliminating the interference of appreciable amounts of bismuth, chromium, ferric iron, and uranium. Among the anions studied, only cyanide, dichromate, nitric, and sulphite interfered appreciably. R. E. S.

Ephedra and Ephedrine in Nasal Sprays, Assay of. Report No. 6 of the Poisons Sub-Committee of the Analytical Methods Committee of the Society of Public Analysts. (*Analyst*, 1948, **73**, 312.) The method of the British Pharmaceutical Codex, 1934, for the determination of the total alkaloids in ephedra was regarded as satisfactory. For the determination of ephedrine in sprays an aliquot portion of the spray is steam distilled in the presence of sodium chloride and sodium hydroxide, the ephedrine being collected in a standard excess of 0.05N sulphuric acid which is titrated against 0.05N sodium hydroxide. The distillation is continued until no further alkaloid is removed and the result is calculated as anhydrous ephedrine. R. E. S.

Ferric Chloride, Reactions of, in presence of Alcohol. L. Rosenthaler. (*Pharm. Acta Helvet.*, 1948, **23**, 271.) It is well known that the blue colour given by phenol with aqueous solutions of ferric chloride does not appear in dilute alcohol. If to 1 vol. of a 1 per cent. solution of ferric chloride

ABSTRACTS

9 vols. of alcohol are added the solution after a time no longer gives the usual reactions of ferric salts. Apparently the whole of the iron is present in the form of a complex, or alternatively in a colloidal form. G. M.

***o*-Hydroxyquinoline Sulphate, Alkalimetric Titration of.** F. Reimers. (*Dansk Tidsskr. Farm.*, 1948, 22, 181.) The pk_2 value for hydroxyquinoline was found to be about 10.6 (in 50 per cent. alcohol) and 11.4 (in 75 per cent. alcohol.) Thus the difference between pk_1 and pk_2 increases with increasing alcohol concentration. In addition, the colour of the titrated solution is brighter in alcohol than in water. The titration may be carried out as follows: 0.1 g. of *o*-hydroxyquinoline sulphate is dissolved in 20 ml. of alcohol (86 per cent. by weight) and titrated with aqueous 0.1 N sodium hydroxide to the colour change of bromocresol purple, or to a green colour with bromothymol blue. This method often gives higher results than bromimetric titration, showing the presence of excess of sulphuric acid. G. M.

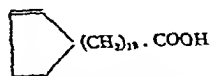
Methylene Blue Periodide as Volumetric Indicator. J. A. Gautier. (*Ann. pharm. Fr.* 1948, 6, 171.) By the addition of iodine and hydriodic acid to methylene blue, a precipitate of the formula $B.HI.2I_2$ is obtained. This reaction may be used both in iodimetry and acidimetry. On the addition of a reducing agent, methylene blue is reformed, and gives a blue colour to the solution, while a larger quantity of a strong reducing agent decolorises the methylene blue forming the leuco base. A suitable indicator may be prepared by mixing a solution of methylene blue (0.0935 per cent.) with an equal volume of 0.01N iodine solution. This suspension should be used fresh. For iodimetry a drop or two is added to the solution being titrated just before the end-point, and titration is continued with thiosulphate until a blue colour appears in the solution. Alternatively, one drop of methylene blue solution may be added, though in this case there is a slight error equivalent to one drop of 0.01N iodine. This method is claimed to be superior to the use of starch. Since the compound is also decomposed by alkalis, it may also be used for acidimetric titrations but in this case it would not appear to offer any advantage over the usual indicators. G. M.

Starch in Plant Tissues, Determination of. G. D. Pucker, C. S. Leavensworth, and H. B. Vickery. (*Anal. Chem.*, 1948, 20, 850.) The method consists of extraction of the starch from a 50 to 250 mg. sample of dried plant tissue with perchloric acid, precipitation with iodine under conditions that have been shown to be quantitative, decomposition of the starch-iodine complex and determination of the sugar produced by hydrolysis of the recovered starch. The results are independent of the composition of the starch of different species with respect to amylose and amylopectin content, in contrast to the colorimetric methods of starch estimation, but once the fundamental values for the starch from a given tissue have been determined in terms of sugar titrations and in comparison with a standard, e.g. a preparation of potato starch, the more rapid colorimetric method can be used in a series of determinations on the same tissue. For a variety of plant tissues results are accurate to within 2 per cent. E. N. I.

FIXED OILS, FATS AND WAXES

***dl*-Hydnocarpic Acid, Synthesis of.** D. G. M. Diaper and J. C. Smith. (*Biochem. J.*, 1948, 42, 581.) The accepted structure assigned to

hydnocarpic acid has been confirmed by synthesis. The ester-acid chloride of sebacic acid reacts with ethyl sodioacetoacetate and the product yields a sodio derivative which reacts in the cold with cyclopent-2-enyl chloride to give a complex; this complex is hydrolysed mainly with the loss of the acetyl and carbethoxy groups to 10-ketohydnocarpic acid.



The keto acid was isolated as the semicarbazone which on heating with sodium ethoxide yielded *dl*-hydnocarpic acid. The synthetic acid did not depress the melting point of the natural *d*-acid and confirmation was obtained from the preparation and identity of the two dihydroderivatives in which there is no asymmetry.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Ascorbic Acid, Pure, Stability of Solutions of, and of Dehydroascorbic Acid. P. Guild, E. E. Lockhart and R. S. Harris. (*Science*, 1948, 107, 226.) Because there appears to be lack of agreement between the content of ascorbic acid in foods as measured by the 2:6-dichlorophenolindophenol method and the 2:4-dinitrophenylhydrazine method, the suitability of these two methods for determining ascorbic or dehydroascorbic acid has been compared. The effects of oxalic acid and metaphosphoric acid on the stability of ascorbic and dehydroascorbic acids in solution have also been investigated. It was found that the 2:4-dinitrophenylhydrazine method gave higher results than the other since the reagent reacts with products related to ascorbic acid which are biologically inactive; this may prove helpful when assaying the original vitamin C content and not the actual content. The method using 2:6-dichlorophenolindophenol gave results which closely resembled the biologically active content of ascorbic acid and dehydroascorbic acid. Ascorbic acid was found to be stable for at least 12 days when kept at 4°C. in a solution containing 5 per cent. of oxalic acid and 10 per cent. of acetic acid; in a solution containing 5 per cent. of metaphosphoric acid and 10 per cent. of acetic acid it was stable for at least 8 days under similar conditions. Dehydroascorbic acid was unstable in neutral solution at room temperature, and neither oxalic nor metaphosphoric acids prevented the irreversible change into biologically inactive forms. For stabilising ascorbic acid, oxalic acid was more effective, more stable, more convenient and less expensive than metaphosphoric acid.

L. H. P.

B₁₂, a New Vitamin of the B Group. G. Fraenkel, M. Blewett and M. C o l e s. (*Nature*, 1948, 161, 981.) The common mealworm, *Tenebrio molitor*, has been shown to be a very suitable test subject for folic acid and certain other, still unidentified, B factors. When grown on an artificial diet consisting of casein, glucose, water-insoluble fraction from yeast, cholesterol, a salt mixture, and aneurine, riboflavine, nicotinic acid, pyridoxine, pantothenic acid, choline chloride and inositol in ample amounts, at least two more factors are essential for growth and survival, namely, folic acid and a factor contained in a norite filtrate from yeast or liver extract; in the absence of both these factors growth is very slow and mortality high. The authors have tentatively named the norite filtrate factor B₁₂ (to indicate its activity on *Tenebrio*). The reported absence of significant amounts of folic acid or the conjugate, pteroylheptaglutamic acid, in well known commercial parenteral liver extracts was fully confirmed in tests with *Tenebrio*.

Further tests to ascertain the possible B₁₂ effects of these extracts, showed no effect with one of the extracts in any concentration, and a positive effect with another extract in only a very large dose. It is obvious that the kind of activity for which the anti-anæmic concentrates have been developed must be entirely different from that of folic acid or B₁₂. S. L. W.

Schiff's Reagent, Use of Thionyl Chloride in the Preparation of. J. C. Barger and E. D. DeLamater. (*Science*, 1948, 108, 121.) 1.0 g. of basic magenta is dissolved in 400 ml. of distilled water, 1. ml. of thionyl chloride is added and the mixture allowed to stand for 12 hours. The decolorised solution is cleared by shaking with 2 g. of charcoal and filtering immediately. Alternatively, the treatment with charcoal may precede the addition of the thionyl chloride. The pH of the resulting solution is 1.24, as opposed to 1.38 for the solution prepared in the usual manner. When used in the Feulgen reaction, Schiff's reagent prepared with thionyl chloride is a successful nuclear stain for fungi, *Blastomyces dermatitidis* and *Saccharomyces cerevisiae*, in addition to tissue sections of human thymus, kidney, liver and spleen. G. B.

Vitamin B₁₂ and Thymidine, for growth of *Lactobacillus lactis*. W. Shive, J. M. Ravel and R. E. Eakin. (*J. Amer. chem. Soc.*, 1948, 70, 2614.) A medium previously described is modified by adding an oleic acid source, tween 80, 10 mg./10 ml., enzymatic digest of casein, 10 mg./ml. or clarified tomato juice, 0.5 ml./10 ml., and Wilson's liver fraction LR, 10 µg/10 ml. and replacing a phosphate buffer by sodium acetate. For the growth of *Lactobacillus lactis* Dormer, thymidine can replace liver extracts containing the principles active against pernicious anaemia, half-maximum growth stimulation requiring 1 to 3 µg./10 ml. Thymine (100 µg./10 ml.) is inactive. Probably vitamin B₁₂ functions in the biosynthesis of thymidine. In the medium containing tomato juice, 1 ml. of aerated water in 10 ml. can replace liver extracts; this effect is enhanced by adding ascorbic acid. Aerated water is not effective in the medium made with enzymatic digest of casein, but 1 mg. of ascorbic acid in 1 ml. of aerated water/10 ml., can replace liver extracts. G. B.

Vitamin D. Potency of the U.S.P. Reference Cod-liver Oil. W. Dasler, C. D. Bauer, and M. van Nostrand. (*J. Lab. clin. Med.*, 1947, 32, 1251.) Three fresh samples of calciferol from entirely different sources, were dissolved in corn oil and repeatedly assayed against the U.S.P. reference cod-liver oil No. 2. The results indicate potency-values of 50 ± 2 units/µg. for all three samples. Similar bioassays of samples of parallel physical and chemical purity made in 1937-1938 against an earlier U.S.P. reference oil gave values of 40 units/µg. This discrepancy in potency-value, confirming repeated observations of recent teams of workers, can only be explained upon the hypothesis that the reference standard cod-liver oil is deteriorating, and should therefore be replaced, as regards vitamin D, by a primary standard, viz., pure crystalline vitamin D₂. F. J. D.

BIOCHEMICAL ANALYSIS

p-Aminosalicylic Acid in Blood and Cerebrospinal Fluid, Determination of. W. Klyne and J. P. Newhouse. (*Lancet*, 1948, 255, 611.) A colorimetric method for the determination of p-aminosalicylic acid in blood and cerebrospinal fluid has been developed. The procedure is as follows:—Add 0.5 ml. of oxalated whole blood or 1 ml. of cerebrospinal fluid to

7 ml. of water, mix until caking occurs, add 3 ml. of 20 per cent. *p*-toluenesulphonic acid, allow to stand for 5 minutes and filter through a No. 40 or 42 Whatman paper. To 5 ml. of the clear filtrate add 1 ml. of citrate buffer solution, 0.75 M, and 2 ml. of 2 per cent. (Ehrlich's) *p*-dimethylaminobenzaldehyde reagent. Read the colour intensity with a photoelectric photometer using a blue filter, e.g., Ilford No. 602. Use a reagent blank consisting of 1.5 ml. of *p*-toluenesulphonic acid, 1 ml. of citrate buffer and 2 ml. of Ehrlich's reagent and made up to 8 ml. with water. A calibration curve is constructed from 3 standards of sodium *p*-aminobenzoate corresponding to 20, 10 and 4 mg. of *p*-aminosalicylic acid per 100 ml. Streptomycin (1000 μ g/ml. of plasma) does not interfere with the estimation but the method cannot be used if other primary aromatic amines are present.

E. N. I.

Salicylates in Blood, Determination of. M. Volterra and D. M. Jacobs. (*J. Lab. clin. Med.*, 1947, 32, 1282.) Salicylates may be determined by a simple and rapid method based on the xanthoproteic reaction, in volumes of 1 ml. of blood serum or plasma, deproteinated by trichloroacetic acid. The yellow colour subsequently developed by the reagents is directly proportional to the concentration of salicylates as observed in recovery-values ranging from 5 to 80 mg. per cent. determined either photoelectrically or by direct vision against a standard series of potassium dichromate units. Good agreement was obtained between the authors' method and that of Coburn for values ranging from 5 to 55 mg. per cent.

F. J. D.

Streptomycin, Identification on Paper Strip Chromatograms. R. E. Horne, Jr. and A. L. Pollard. (*J. Bact.*, 1948, 55, 231.) A paper strip chromatographic method is described for detecting the presence of streptomycin in culture filtrates, etc., using 3 per cent. ammonium chloride solution as the solvent, the mechanism involved being a salting-out process. The paper strips are spotted near one end with the solution under test and the "spot" dried. They are then suspended, spotted end downwards, so that the lower ends are immersed in the ammonium chloride solution, the whole being placed in a closed container so as to maintain a saturated atmosphere. The solvent moves the streptomycin in a sharp band near the advancing solvent front. After 4 to 12 hours, when the solvent front has reached the desired height, the strips are dried and developed by means of a modification of the Sakaguchi reaction. The dry strip is sprayed with N/2 sodium hydroxide and immediately with 0.25 per cent. α -naphthol. After 2 minutes it is sprayed with sodium hypochlorite prepared as described by Sakaguchi. Streptomycin is shown as a brilliant red band.

H. T. B.

Vitamin A, Simultaneous Comparative Carr-Price Reactions for Determination of. W. Koch and D. Kaplan. (*J. biol. Chem.*, 1948, 172, 363.) The difference in rates of fading of the Carr-Price colour of graded concentrations of standard vitamin A, provides the basis of a photoelectric method in which standard and "unknown" are matched simultaneously thereby cancelling errors. Initially, the currents generated by two photocells receiving the light from two reaction tubes containing two different concentrations of vitamin A in Carr-Price reagent, were balanced on a galvanometer sliding bridge scale. When the differences in fading-rates in seconds, for graded concentrations of vitamin A standard in U.S.P. units were plotted against bridge-readings, it was observed that with falling concentrations the curves

flatten, change their slope and (at 10 units) approach the 80 per cent. transmission line, used as a convenient arbitrary standard. Subsequently, a calibration diagram was constructed by joining each of the predetermined bridge readings equivalent to fading values at 15 to 180 sec. in 15 sec. intervals, for concentrations of 50, 40, 30 and 20 units respectively, to the point of intersection of the 10 unit abscissa with the arbitrary "80" bridge ordinate. Determination of "unknowns" were then made by entering each bridge-reading as a dot on the appropriate seconds line, tracing the best fitting line through the points back to the abscissa, and reading the vitamin A in units/ml. Evidence is offered that values obtained by this method are in fair agreement with figures measured by ultraviolet absorption.

F. J. D.

PHARMACY

DISPENSING

Oils and Fats, Sterilisation of. H. Hurni. (*Pharm. Acta Helvet.*, 1948, 23, 283.) A number of methods are given in various pharmacopœias for the sterilisation of oils and fats. The ordinary conditions of sterilisation in an autoclave do not apply since water is absent, and in fact bacteria are killed in the same time at any temperature, whether they are in fat or in dry air. It has been shown that fats and oils as used for pharmaceutical preparations are nearly always sterile. Suitable methods for sterilisation are as follows: 4 hours at 140°C.; 3 hours at 145°C.; 2 hours at 150°C.; or filtration through a Berkefeld filter at 80° to 90°C. The Seitz filter is not effective for this purpose.

G. M.

Vitamin A, Stability of, In Pills. P. T e r p. (*Arch. Pharm. Chemi.*, 1948, 55, 513.) Pills were prepared according to the following two formulæ: I. lactose-starch granulate, 54 g.; vitamin concentrate, 8.4 g.; hydroquinone, 0.03 g.; aluminium oxide anhydrous, 9.6 g.; II. granulate, 41.4 g.; vitamin concentrate, 9 g.; hardened mustard oil, 12 g.; hydroquinone, 0.06 g.; aluminium oxide, anhydrous, 15.6 g. Both of these lost 40 per cent. in strength in 3 months. A further batch was made as above, with the addition of 0.25 per cent. of hydroquinone added to the granulate during granulation. These pills became black after a few days, and the vitamin was almost completely destroyed in a week. A number of pills were then made by the dropping method in which the molten mixture is solidified by dropping into cold alcohol (d. 0.883). Good results were obtained with the following formula: vitamin A concentrate (160,000 units/g.) 1 part; hardened arachis oil 8 parts; hydroquinone, 0.25 per cent. These pills showed a loss in strength of 12 per cent. after keeping for 12 months at ordinary temperature, and no loss over the same period in an ice chest.

G. M.

GALENICAL PHARMACY

Penicillin Depot Preparations. J. Büchi and F. O. Gundersen. (*Pharm. Acta Helvet.*, 1948, 23, 290.) Measures found effective for delaying the absorption of penicillin were: to avoid aqueous solutions, to surround the solid penicillin or sparingly soluble penicillin salt with an oil base, to choose the optimum degree of fineness, and to add substances (wax, adrenaline, aluminium stearate) which delay absorption. Certain proprietary preparations were effective, so that with these only one injection daily was necessary. Of the formulæ given, the following is the best; with an

GALENICAL PHARMACY

injection of 1 ml., the effect lasts for more than 12 hours; and with 2 ml. for 24 hours; benzylpenicillin sodium cryst., 300,000 units; adrenaline, 0.3 mg.; sterile neutralised olive oil, to 1 ml. G. M.

Tincture of Iodine, Stability of. A. Tennoe. (*Dansk Tidsskr. Farm.*, 1948, 22, 226.) An alcoholic solution of iodine, without potassium iodide, rapidly decomposes, and after 1 week's storage it already contains an amount of hydriodic acid greater than the limit allowed by the Danish Pharmacopœia (i.e. 0.13 per cent.). The reaction is apparently reversible, since if the preparation, after heating at 90°C, is then kept at ordinary temperature for some time, the acidity decreases and the free iodine increases. After 12 weeks' storage at 20°C., there was no appreciable difference between two preparations containing respectively 5.06 per cent. of iodine with 3.54 per cent. of potassium iodide, and 5.04 per cent. of iodine with 2.08 per cent. of potassium iodide. When kept at 90°C. for 4 weeks, the preparation with the smaller quantity of potassium iodide showed 50 per cent. more acidity than the other. G. M.

PHARMACOGNOSY

Hydrastis, Histological Peculiarities of its Adulterants. R. Lemesle. (*C. R. Acad. Sci., Paris*, 1948, 227, 686.) Blaque and Maheu (Rev. gén. Bot. 1947, 54, 138) have described the peculiarities of the pith and pericycle of the rhizomes of *Xanthorrhiza apiifolia* L'Hér. and of *Coptis Teeta* Wall, which are adulterants of *Hydrastis canadensis* rhizome. In this paper the xylem elements of these three rhizomes are described. *Hydrastis* rhizome has vessels with bordered pits, containing pectosic mucilage and of diameter 36 to 44 μ . Wood parenchyma and fibres (without starch) are also present. In *Xanthorrhiza* the vessels are up to 55 μ in diameter and have no bordered pits; parenchyma is absent. The fibres contain numerous spherical or polyhedral starch granules (4 to 6 μ). In *Coptis*, narrow tracheidal vessels, such as occur in ipecacuanha, are present, maximum diameter 18 μ . Parenchyma is absent and the fibres contain starch granules. J. W. F.

PHARMACOLOGY AND THERAPEUTICS

Caronamide, Effect on Penicillin Plasma Concentration in Children. M. Rapoport, F. B. Corneal, K. H. Beyer and W. F. Verwey. (*Amer. J. med. Sci.*, 1948, 215, 514.) Oral administration of caronamide to children, in a dosage of 0.2 g./kg. of body weight per day, together with penicillin increased the concentration of penicillin in the plasma to from 1.8 to 2.8 times the control values obtained with penicillin alone. With a caronamide dosage of 0.4 g./kg. of body weight per day the penicillin concentration was increased by from 2.8 to 14.5 times the control concentration. Toxic symptoms during 1 to 2 weeks administration were not sufficient to warrant discontinuance of caronamide treatment. Renal function tests before and after treatment showed that the drug does not irreversibly affect kidney function. H. T. B.

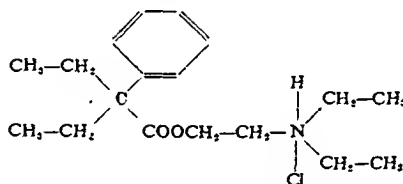
Cuprelone, Trigeminal Neuralgia Treated With. A. M. G. Campbell. (*Lancet*, 1948, 255, 690.) Cuprelone, cupro-allyl-thiourea-sodium benzoate, contains 19 per cent. of copper. It was introduced as a non-toxic substitute for gold compounds. It is supplied in dry ampoules, containing 10 to 100 mg. and is dissolved in sterile distilled water for intravenous

ABSTRACTS

injection; 100 mg. produced a rise in blood-copper level for 2 hours after injection. All the extra copper is probably excreted during the next 2 or 3 weeks. It was administered in 13 cases of trigeminal neuralgia and in some the pain was relieved; its toxic effects were negligible. H. F.

Dipole Moment and Physiological Activity. B. Melander. (*Farm. Revy*, 1948, 47, 503.) In the hexachlorocyclohexane series, maximum insecticidal activity is shown by the isomer with a dipole moment in the region of 4D; the two isomers with no dipole moment are inactive. A similar effect is observed with the same compound in the production of total colchicine mitosis in *Allium Cepa*. Local anæsthetic bases (procaine and xylocaine) have a moment about 4D, whereas the group of aliphatic alcohols and benzyl alcohol have moments 1.7 to 2 D. The latter group, unlike the former, do not produce convulsions when injected intravenously. Ether and chloroform have dipole moment 1.15 D. G. M.

Parpanit, Treatment of Parkinsonism with. W. F. Dunham and C. H. Edwards. (*Lancet*, 1948, 255, 724.) The effects of parpanit, a synthetic compound, closely related to trasentin and pethidine (dolantin), and having the structural formula



were investigated in 25 cases of Parkinsonism, in all of which treatment with solanaceous drugs had been given for more than a year. The results showed no striking difference from those obtained with solanaceous drugs. The optimum individual dose varied from 0.025 to 0.1 g., usually 3 to 4 times a day. The side effects of parpanit, although similar to those of the solanaceous drugs, differed sufficiently in intensity and frequency to make it the drug of choice for some patients. E. N. I.

Penicillin Aluminium Salt in Mouse Protection Tests. R. D. Reid. (*Proc. Soc. exp. Biol. N.Y.*, 1947, 66, 605.) Aluminium penicillin is a water-insoluble salt which has been administered in arachis oil suspension. A dose of 300,000 I.U. given intramuscularly to human subjects has been found to give blood levels of 0.03 I.U./ml. for 12 to 24 hours. The author compared the dosage required to give a survival rate of 50 per cent. in mice infected intraperitoneally with 1000 M.L.D. of a diluted culture of *Diplococcus pneumoniae* Type 1, with the dosage of sodium penicillin in oil and wax and calcium penicillin in oil, giving injections for 14 days. The figures obtained were respectively 34 units, 40 units and 95 units. H. T. B.

Penicillins F, G, K and X; Relative Antisymphilitic Activities. H. Eagle and R. Fleischman. (*J. Bact.*, 1948, 55, 341.) The antisymphilitic activities of penicillins F, G, K and X, and of bacitracin were evaluated by a method based on the fact that an extremely small amount of treatment is sufficient to terminate syphilitic infection in rabbits, provided it is administered soon after inoculation and before the appearance of the primary lesion. Rabbits were inoculated intradermally with 2,000 *Treponema*

pallidum, and treated 4 days later with intramuscular injections of the various penicillins or bacitracin, repeated once daily for 4 days. It was found that penicillins F, K and X were approximately 8, 12 and 14 per cent. respectively as active as penicillin G, and a crude preparation of bacitracin assaying at 30 units/mg. was 10 per cent. as active. Comparison of the results obtained with those of other workers shows that the absolute and relative activities of the various penicillins and of bacitracin vary widely according to the method of assay.

H. T. B.

Penicillin Suspension with Adrenaline for Gonorrhœa. A. Cohn and B. A. Kornblith. (*Amer. J. med. Sci.*, 1948, 215, 506.) A total of 300 male ambulatory patients with acute gonococcal infections were treated intramuscularly or subcutaneously with a single dose of suspension of potassium penicillin in oil containing adrenaline. The suspension contained 300,000 units of potassium penicillin and 0.3 mg. of adrenaline in 1 ml. of vegetable oil, and dosage varied between 0.25 and 1 ml. Only exceptionally were 2 ml. doses used. Single injections of 150,000 units cured 97 of 100 patients treated, the criteria of cure including a bacteriological and clinical check for 2 or 3 weeks after treatment. The other 3 cases were promptly cured by a second injection of the same amount, indicating that the relapse strain had not become resistant to penicillin. All of 19 patients treated with 200,000 units in 1 ml. intramuscularly were cured. A table gives the results obtained on 154 of the patients treated, the remainder of the 300 not attending for final determination of cure. No untoward local or systemic reactions were observed.

H. T. B.

Salicylazosulphapyridine, Therapeutic Action of. N. Svartz. (*Bull. schweiz. Akad. med. Wiss.*, 1948, 3, 311.) It has been shown that all acid azo compounds have a marked affinity for connective tissue, particularly tissue rich in elastin; and their localisation in such tissues may be detected microscopically by the fluorescence. Such compounds should therefore be effective in ulcerative colitis and rheumatic polyarthritis. Cases of colitis were treated usually with 1 g. of salicylazosulphapyridine 6 times a day, the dose being decreased as the symptoms improved. It was found necessary to continue the treatment over a long period with 0.5 g. 2 to 3 times a day. Results of 119 cases show a considerable improvement or cure in 108 of them. For acute polyarthritis, 100 patients were treated (some with salicylazosulphathiazole) in the period 1941 to 1945. In 1947, 92 were reported free from symptoms. The results for chronic polyarthritis were less satisfactory, only about 40 per cent. showing any considerable improvement. Although the administration was generally *per os*, periarticular injection was sometimes useful.

G. M.

Streptomycin, Effects on *Mycobacterium tuberculosis* Infection by Inhalation. C. Levaditi, A. Vaisman and P. Levy. (*C. R. Acad. Sci., Paris*, 1948, 227, 987.) Mice were infected by injection with *Mycobacterium tuberculosis*, human strain H 512, and 30 were kept in an atmosphere containing streptomycin introduced under a pressure of 0.5 kg. for 6 hours a day for 6 weeks. The total amount of drug evaporated during this period amounted to 22 mega-units. Another batch of 20 mice were infected and kept as controls without treatment. Of these all died within 29 days. Of the mice treated with streptomycin, one died on the 46th day, the remainder were then destroyed and examined. From the results it appeared that streptomycin undoubtedly exerted therapeutic activity.

ABSTRACTS

although it was lower than that showed by the subcutaneous injection of 1,000 to 2,000 units daily.

H. F.

Tetra-ethyl pyrophosphate in Myasthenia Gravis. A. S. V. Burgen, C. A. Keele and D. McAlpine. (*Lancet*, 1948, 254, 519.) T.E.P.P. was given to 3 patients either as a 0.5 per cent. solution in propylene glycol, intramuscularly, or as a 2 or 5 per cent. solution in propylene glycol, orally. By injection, it was found to be from a third to half as potent as prostigmine but its action lasted about twice as long. Side effects, similar to those produced by prostigmine, were experienced, but the action on the gut producing colic and diarrhoea was prevented by atropine.

G. R. B.

BACTERIOLOGY AND CLINICAL TESTS

Aerosol OT, Synergistic Effect of, on Certain Germicides. G. V. James (*J. Soc. chem. Ind., Lond.*, 1948, 67, 336.) Rideal-Walker coefficients for dispersion of certain germicides in a 20 per cent. solution of a neutral castor oil soap show the following increases on the addition of 0.1 per cent. w/v of aerosol OT (di-octyl-sodiumsulphosuccinate):—phenol (1 per cent.), 0.6; cresol (1 per cent.), 0.9; *p*-chloro-*m*-xylenol (2 per cent.), 1.1; butylphenol (2 per cent.), 1.1; benzylcresol (2 per cent.), 1.6; cresantol-15 (3 per cent.), 2.2. Similar results are obtained with 0.4 per cent. of a commercial product described as the *bis*-ester of sodium sulphonated dicarboxylic acid. The increased germicidal activity appears to be stable over at least 4 months at room temperature. The use of sulphonated castor oil instead of castor oil soap results in a smaller enhancement of R.W. co-efficient. *Bacillus typhosus* was used as the test organism and 0.02 ml. pipettes replaced platinum loops to prevent errors due to differences in surface tension.

G. B.

Penicillin: Induced Resistance and Oxygen Utilisation. W. D. Bellamy and J. W. Klimek. (*J. Bact.*, 1948, 55, 147.) The observation that penicillin-resistant staphylococci grow more slowly than the parent strain, and almost exclusively at the surface of broth cultures, was confirmed on a penicillin-sensitive strain of *Staphylococcus aureus*, and a penicillin-resistant variant of this strain. Growth curves under aerobic and anaerobic conditions showed that the penicillin-resistant variant grew more slowly than the parent-sensitive culture, and had lost the ability to grow anaerobically. Strains of *Streptococcus faecalis*, *Strep. mastitidis* and *Clostridium welchii* when treated in a similar manner failed to develop resistance to penicillin. It is suggested that the development of resistance is dependent on the power to grow under aerobic conditions.

H. T. B.

Penicillin-Resistant Staphylococci. W. D. Bellamy and J. W. Klimek. (*J. Bact.*, 1948, 55, 153.) The properties of penicillin-resistant variants of *Staphylococcus aureus* have been compared with those of the parent sensitive culture. The variant was 60,000 times more resistant to penicillin than the original culture, was Gram-negative, and had lost the ability to grow anaerobically. Increase in resistance is accompanied by a progressive loss of fermentative activity, but the resistant variant can synthesise nicotinic acid in quantities sufficient for growth. It produces an extracellular penicillinase when grown in the presence of penicillin. Serial transfers through a deficient medium will cause a reversion from Gram-negative rods to the original Gram-positive staphylococcus forms which have lost most of their resistance.

H. T. B.

BOOK REVIEWS

THE UFAW HANDBOOK ON THE CARE AND MANAGEMENT OF LABORATORY ANIMALS, with an Appendix on Statistical Analysis. Edited by A. M. Worden. Pp. 368, 70 Figs. Bailliere, Tindall and Cox, London, 1947, 31s. 6d.

This handbook has been produced by the Universities Federation for Animal Welfare. It is intended as a practical introduction to the husbandry of laboratory animals for use by research workers and technicians. Both novices and experienced workers in fields involving the keeping of health stocks of animals for experimental purposes will find much of value to them in this book. The novice may be bewildered by the numerous variations in details which he will find reported from different laboratories. The experienced worker will be interested to learn what variations of detail have been found valuable by other workers. Scarcely any detail can be considered very much better than the rest and much depends on the availability of varieties of such things as food pots and water bottles, material for constructing cages and stands for cages, etc. One general principle, however, stands out pre-eminently, the simpler the arrangement the better. Simplicity in racks for holding cages, simplicity in the construction of the cage itself, simplicity in the food pots and water bottles all help towards cleanliness. Ledges in racks harbour dust and food, and getting it out with a brush spreads most of it into the air again to settle elsewhere. Labour and time are wasted. The best arrangement seen by the reviewer is that in her own laboratory, viz., racks made of gas piping, which, being cylindrical, affords the smallest possible area for collecting dust, and cages (not in her own laboratory) made of sheets of wire mesh with strong borders (for floor, walls and ceilings), held together with movable clips. These cages are hooked on the bars which hold the trays of the cages above, themselves loose and easily pulled out for cleaning. Each cage can be taken to pieces in nearly no time and many more cages can be sterilised at one time when in pieces than when whole. However, "chacun à son goût" (and his pocket). The animals considered in detail in this book are the rabbit, guinea-pig, Norway rat, black rat, mouse wild house-mouse, wood mouse, deer mice, cotton rat, common or field vole, Orkney vole, golden hamster, ferret, hedgehog, pigeon, canary, amphibia, *Xenopus laevis* Daudin, and fresh water fish. Others dealt with more lightly are anthropoids, dogs and cats, horses, other ungulates, shrews, vampire bats, poultry, reptiles, marine forms and other vertebrates. Housing, nutrition, breeding, etc., and common diseases of each species are dealt with. In addition there are useful chapters on Law and Practice: The Rights of Laboratory animals, (much of which will be news to most animal workers), Pests of the Animal House and their Control, and an Appendix of 70 pages, a conspectus of the Elements of Statistical Analysis which should be read and digested if possible even by those readers who are already familiar with the recommended ways of planning and assessing the results of experiments. Another man's way of putting it is always worth examining.

K. H. COWARD.

LETTERS TO THE EDITOR

The Estimation of *d*-Tubocurarine Chloride

SIR,—A method for the estimation of *d*-tubocurarine chloride, suitable for standardisation of injections, depending on the development of a blue colour with Folin-Ciocalteu phenol reagent, has been described by Foster¹, and its application in the assay of the total quaternary alkaloids in crude curare has been described by Foster and Turner². In our hands this method has not been found to give results more accurate than the ± 5 per cent. claimed, and, as stated, it is certainly necessary in all cases to prepare a standard at the time the assay is made. The issue of the Therapeutic Substances Amendment Regulations, 1948, has emphasised the necessity for accurate control of this preparation. We have found the qualitative colour test given in these regulations—the development of a cherry-red colour when a few crystals of the substance are added to 0.5 ml. of Millon's reagent—to form the basis of a suitable colorimetric assay, accurate to ± 2 per cent. and requiring less manipulation than with the Folin-Ciocalteu reagent.

To prepare Millon's reagent pure mercury is dissolved in twice its weight of nitric acid B.P., heating if necessary to complete solution. The solution is diluted with twice its volume of water, allowed to stand overnight and filtered if necessary. Standard colour solutions are prepared as follows. A standard 0.1 per cent. solution of *d*-tubocurarine chloride in water is prepared. 1, 2, 3, 4 and 5 ml. portions of this solution are diluted to 10 ml. with water and 5 ml. of Millon's reagent is added to each. A blank is prepared from 10 ml. of water and 5 ml. of reagent. The contents of each tube are thoroughly mixed and allowed to stand at room temperature for 2 hours (Unless a standard solution is set up side by side on each occasion a test is made, it is desirable to standardise a temperature, e.g., $20 \pm 1^\circ \text{C.}$, at which colour development is allowed to proceed.) Maximum colour development is attained at this time, the variation in colour for a few minutes on either side being negligible. Shortly after 2 hours precipitation commences in the more concentrated solutions. Light transmission is measured on a suitable photoelectric absorptiometer, using a cell having a light path of 2 cm. and a filter with a maximum transmission at about 430 μ . A straight line graph is obtained by plotting the logarithmic (density) readings against concentration. For the assay of a sample 2 mg. is a convenient quantity to use and a volume containing this amount is diluted to 10 ml. with water and 5 ml. of reagent added. After standing at room temperature for 2 hours, the colour is compared against a blank, which should contain in similar proportions any additional solvent known to be present in the test sample.

Ethyl alcohol, benzyl alcohol, glycerol and sodium metabisulphite, any of which may be encountered in injections, do not interfere with the development of the colour, and it is unlikely that any inorganic salts which may be used as buffers, or to prepare isotonic solutions, will affect the results. Phenol and chlorocresol interfere, producing a pink colour similar to that given by the alkaloid, and may be removed prior to colour development by extraction with chloroform, as described by Foster.¹

Duncan, Flockhart and Co., Ltd., Edinburgh.
January 19, 1949.

A. M. PRYDE,
F. R. SMITH.

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2. Foster and Turner, *Quart. J. Pharm. Pharmacol.*, 1947, 20, 228.

NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Cremosuxidine* is a suspension of succinylsulphathiazole 10 per cent., with pectin 1 per cent. and kaolin 10 per cent. It is indicated in the treatment of specific and non-specific diarrhœas, including bacillary dysentery, paradysentery, salmonellosis, diarrhœa of the new-born and summer diarrhœa; it is also of value in other conditions where a liquid form of succinylsulphathiazole is preferred to powder or tablets. The dose for adults is 2 to 3 tablespoonfuls 4 times daily, for children 1 to 2 tablespoonfuls 4 times daily, and for infants 2 to 3 teaspoonfuls 4 times daily, these doses being equivalent to a total daily dose of succinylsulphathiazole of 12 to 18 g. for an adult, 6 to 12 g. for a child, and 3 to 5 g. for an infant. For infants, the suspension may be incorporated with milk, or the infant's formula, up to 50 per cent. of the quantity of the feed, and will still pass through an ordinary teat. Cremosuxidine is supplied in bottles containing 4 or 16 fl. oz. S. L. W.

Etamon* is a proprietary form of tetraethylammonium chloride, a quaternary ammonium compound. It is indicated in the treatment of thromboangiitis obliterans, peripheral arteriosclerosis obliterans, thrombophlebitis, causalgia, and functional vascular disorders such as Raynaud's syndrome. It may also be employed diagnostically in many acrovascular conditions, and in neurogenic hypertension, to determine the contribution of sympathetic stimuli in the maintenance of vasospasm. The injections may be given either intravenously, in a dose of 1 to 5 ml., but not exceeding 7 mg./kg. of bodyweight, or intramuscularly, in a dose of 10 to 12 ml., but not exceeding 20 mg./kg. of bodyweight, the frequency of injections depending on the duration or relief of symptoms. It should be used with caution in patients with severe hypertension, especially in the presence of poor renal function or high diastolic pressure. It should not be used in cases with a recent coronary thrombosis, and only with caution in elderly and arteriosclerotic patients. It is issued in rubber-capped bottles containing 20 ml. of a sterile 10 per cent. aqueous solution. S. L. W.

Ferrivenin* is a preparation of saccharated iron oxide for intravenous injection, 5 ml. of a 2 per cent. solution containing 100 mg. of Fe. It is especially indicated in the treatment of refractory hypochromic anæmias. The total dose of ferrivenin required for the individual patient may be calculated by relating the hæmoglobin deficit of the patient to the fact that 25 mg. of iron are required to produce a 1 per cent. rise in hæmoglobin. A test dose of 1.5 ml. is usually given intravenously on the first day, followed by an injection of 3 ml. on the second day, and subsequent injections of 5 ml. daily or on alternate days until the course is completed. The injections should be given slowly and care taken to avoid perivenous leakage. Alternatively, the total required dose may be given by a single intravenous infusion. It is claimed that the injections are painless and non-toxic and do not give rise to thrombosis or embolism, and that the usual symptoms of nausea, indigestion, anorexia and diarrhœa, or constipation, associated with the oral administration of iron, do not occur. Ferrivenin is issued in ampoules containing 5 ml. of a 2 per cent. solution. S. L. W.

NEW APPARATUS

A LOW PRESSURE HYDROGENATOR OF WIDE APPLICATION

BY A. L. GLENN

From the Pharmaceutical Chemistry Research Laboratories, the School of Pharmacy, University of London

Received January 28, 1949

A NUMBER of low-pressure catalytic hydrogenators have been described in the literature, most of them being intended for use in the determination of structure, in which the hydrogen absorbed by small quantities of material must be measured with great accuracy^{1,2}. However, such apparatus is quite unsuitable for normal laboratory-scale hydrogenations. The small number of hydrogenators intended for synthetic work vary from simple devices^{3,4,5,6}, to those which are exceedingly complex⁷. The simpler designs are divisible into two groups; one kind will handle large volumes of hydrogen with ease and fair accuracy of measurement, but is somewhat inaccurate for quantities of 1 litre or less^{5,8}. The other type is capable of dealing with small quantities with good accuracy, but requires too much refilling when large volumes are required^{3,4,6}.

In the present design an attempt has been made to produce a robust apparatus having as many advantages as possible compatible with simplicity of construction and safe operation; its main features are shown in the theoretical diagram (Fig. 1). The apparatus is easily moved from bench to

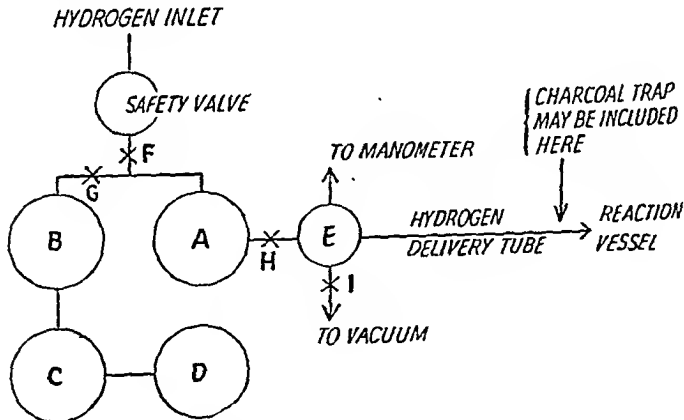


FIG. 1.—Theoretical diagram \times = tap

bench, since it has been built up on a laboratory trolley. Filling is almost instantaneous, for there is no large volume of liquid to displace, a decided advantage during large-scale hydrogenations. The hydrogen uptake is measured by means of a manometer in conjunction with a reservoir of known volume; this reservoir may have a capacity of either 1 or 4 litres, according to the position of a tap at the back of the apparatus. When the reservoir capacity is 1 litre, the delivery of a few hundred ml. of hydrogen results in a considerable fall in the manometer reading, which makes accurate measurement an easy matter. The 4-litre capacity is more convenient for larger volumes. During a series of small-scale hydrogenations, using the

1-litre capacity, the rest of the reservoir may be used for hydrogen storage, thus making the apparatus fairly independent of a cylinder.

The manometer has been placed in circuit at such a point that on closing the reservoir outlet tap the absorption rate, as measured by the manometer, is magnified. This interferes in no way with the initial and final measurements of the reservoir pressure, and is most useful for observing the rate of hydrogen absorption. The maximum pressure obtainable is about 2 atmospheres, but the apparatus will also operate at pressures below 1 atmosphere, a fact which may prove useful in the controlled hydrogenation of substances which absorb hydrogen with great vigour. In order to safeguard the operator, a safety valve has been incorporated, which cannot fail to leak above a predetermined pressure. An efficient charcoal trap has been included for use on those occasions when it is thought that catalyst poisons are being introduced either from the hydrogen used or from the apparatus itself.

The Reservoir.—This comprises four 1-litre bolt-head flasks, A, B, C and D, each flask being closed by a well-fitting rubber bung, through which the necessary connecting tubes pass.

After wiring in the bungs, each flask is wrapped in cotton material and secured to a cork ring by means of insulation tape, so that the flasks are protected during movement of the apparatus. The four flasks are mounted on the lower tier of the trolley and are kept in fairly rigid and symmetrical arrangement by means of a length of brass strip, which is looped around each flask neck, and serves to keep the flasks about three inches apart. The reservoir compartment is covered in with thick plywood in order to protect the operator, whilst tap G, which controls the reservoir capacity, is mounted so that it projects through a hole in the board at the back of the reservoir compartment. The reservoir inlet and outlet taps, F and H, are mounted onto a plywood frame, screwed to the back of the manometer upright (Fig. 2). This reservoir system may be replaced with steel flasks if required.

The Junction Tube.—The reservoir outlet tap, H, leads to a junction tube, E, in order to effect the connections referred to in the theoretical diagram. This consists of the open end of a thick-walled Pyrex boiling tube joined on to a length of 12 mm. tubing. Three side arms of the same tubing are then blown into the body of the boiling tube. The junction tube is situated in the reservoir

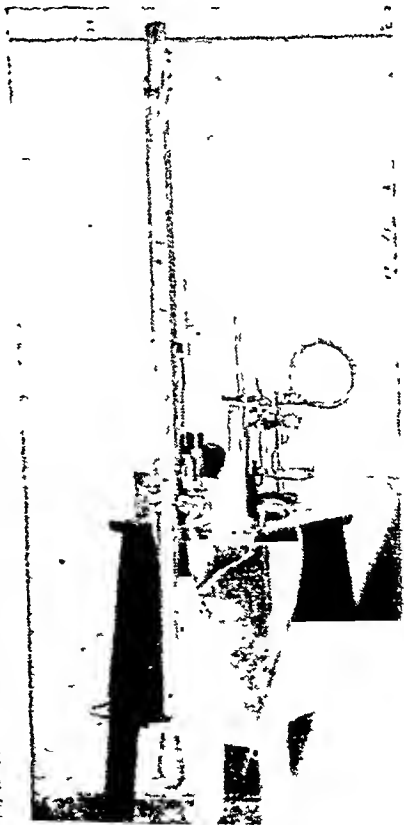


FIG. 2.—General view of apparatus

compartment, whilst the exhaust tap, I, is mounted in a rubber bung, which is fitted into a hole on the top of the trolley.

The Manometer.—An upright of oak ($\frac{1}{8}$ " \times $1\frac{1}{2}$ ") screwed to the edges of the upper and lower tiers supports a metre scale, which slides up and down the upright between two accurately fitted wooden rails. The manometer itself consists of two lengths of thick-walled capillary tubing (internal diameter = 1.5 mm.; external diameter = 6.5 mm.) joined together at the bottom of the "U" by means of a short length of pressure tubing. The use of pressure tubing in this case not only simplifies construction of the manometer and renders it less fragile than an all-glass U-tube, but enables one to vibrate the mercury column before reading to ensure that tailing has not distorted the levels. The two limbs of the manometer are fixed to the rails on the upright by loops of copper wire at intervals; the wire passes around the back of the upright, but not across the metre scale. In filling the manometer air bubbles should be removed by forcing the mercury into the open limb and pushing a long length of copper wire down the tube.

The Safety Valve.—The principle of operation is as follows (see Fig. 3). As the pressure in the flask, L, increases, mercury is forced into the vertical

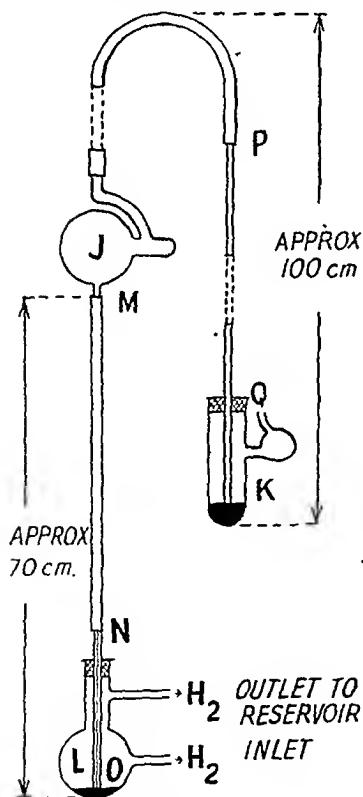


FIG. 3.—Diagram of safety valve

capillary tube OM; the quantity of mercury initially in L is adjusted so that, when the pressure has risen to about 2 atmospheres, the whole of the mercury is in the vertical tube. A further small increase in pressure then forces the mercury into the baffle chamber, J. Hydrogen escapes through the outlet tube K, which contains a small quantity of mercury to act as a seal, during evacuation of the apparatus; the outlet of K is fitted with a miniature mercury baffle. During this process the pressure in the apparatus falls rapidly, and the hydrogen supply must be cut off completely before the mercury in J will again return to the flask, L. Both L and J are constructed from 50-ml. Pyrex flasks, J being the most simple and efficient mercury baffle of several types tried. The tube ON has the same dimensions as that used for the manometer and passes to the bottom of the vessel, L, which is mounted on the bottom of the reservoir compartment as near to the manometer upright as possible. Connection between ON and J is made with a length of capillary pressure tubing, MN; this should be kept reasonably straight. The use of narrow-bore tubing in this part of the valve reduces the volume of mercury

required, and hence lessens the problem of baffling; the baffle, J, is mounted on a bracket, screwed to the back of the manometer upright. After J, ordinary pressure tubing is used in order to reduce the resistance to hydrogen

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flow; PQ is a length of 6 mm. tube, which passes to the bottom of tube K: the latter is mounted on the top of the trolley immediately behind the manometer upright. The introduction of the correct volume of mercury is an

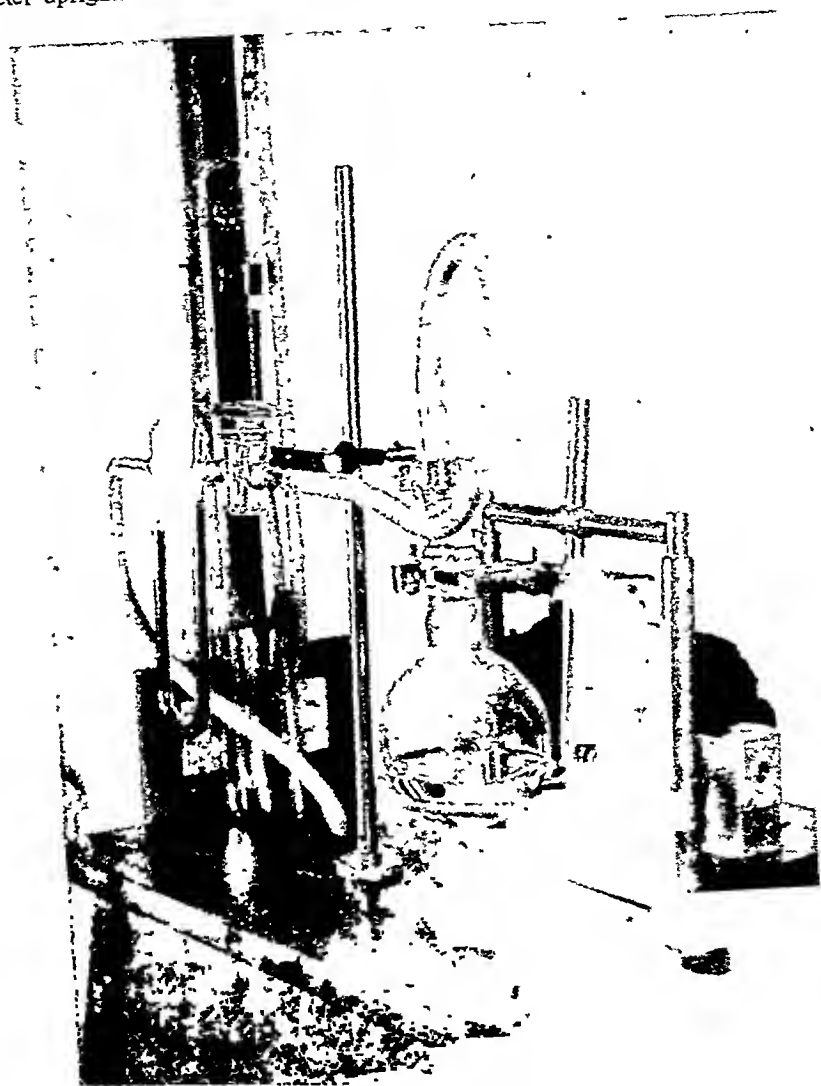


FIG. 4.—Close-up view of apparatus

easy matter; after removing the pressure tube from the top of J, small quantities of mercury are poured in until by trial and error the valve is found to operate at the desired pressure.

The Charcoal Trap.—When immersed in an acetone/solid carbon dioxide bath, this trap is capable of adsorbing 1 litre of hydrogen sulphide: it is therefore a useful adjunct where it is desired to rigidly exclude any catalyst poisons arising from the apparatus or the hydrogen supply. The construc-

tion should be apparent from Figure 4; the U-tube is filled with granular charcoal to give a total path of about 30 cm. length, and a glass wool plug is present at each end to prevent movement during the flow of gas. After use, immerse the trap in boiling water and pass a slow stream of hydrogen through for about an hour. At the end of this operation the trap should be quite free from moisture, otherwise it may block during the next hydrogenation.

The Shaker.—This was specially designed for the apparatus by Dr. W. J. Arrol. The 1/25th h.p. motor is suitably geared down to drive a rocking retort-stand by means of a shaft, the reaction flask being attached to the stand. The principle should be apparent by reference to Figure 4. The dimensions have been carefully worked out, so that the catalyst is very well shaken without undue splashing of the reaction mixture into the neck of the flask. This shaker appears to be much more satisfactory for this purpose than any previously encountered.

General Notes on Construction.—Most of the connections have been made with Portex Plastic Commercial Tubing No. 6c, and the associated glass work has been made from Pyrex tubing of 12 mm. external diameter, with which the plastic tubing forms a very tight-fitting joint. All glass junctions are narrowed at the tips and well-rounded off to facilitate the fitting of the plastic tube. In addition, each connection is doubly wired with No. 18 S.W.G. copper wire. When operating at 60 cm. of mercury above atmospheric pressure, the leakage rate of the whole apparatus is of the order of about 2 mm. an hour; for the 1 litre reservoir capacity this is equivalent to a leakage of approximately 3 ml. of hydrogen an hour, which may be ignored during any but the slowest hydrogenations. When desired, the leakage rate can be reduced further by working at a few cm. of mercury above atmospheric pressure.

The Taps.—Figure 5 shows the way in which high vacuum taps have been adapted to withstand pressure. The taps are well greased with Apiezon "L" and a slotted brass disc seated upon a rubber washer is wired into place, so that the tap is held firmly into its socket. This method has given very little trouble.



FIG. 5.—Close-up view of a tap

Calibration.—It is necessary to know the precise volumes of the reservoir when set at nominal capacities of 1 and 4 litres respectively. The apparatus should be filled with hydrogen and the drop in manometer pressure noted during the delivery of 500 ml.; a volumetric flask and a trough of water at room temperature is useful here. The volumes are then readily obtainable after correcting for aqueous vapour pressure. It is useful for practical purposes to calculate approximately the fall in manometer pressure corresponding with the delivery of 100 ml. of hydrogen in the case of both reservoir capacities. As will be seen below, in calculating the manometer drop for a given hydrogenation it is necessary to add the volume of

pressure corresponding with the delivery of 100 ml. of hydrogen in the case of both reservoir capacities. As will be seen below, in calculating the manometer drop for a given hydrogenation it is necessary to add the volume of

NEW APPARATUS

the dead space in the reaction vessel to the reservoir volume before arriving at the final volume for calculation purposes

Calculation of Reservoir Volume.—Suppose 500 ml. has been collected over water at room temperature.

Let v = volume of reservoir in ml.

m_1 = initial manometer reading in mm.

m_2 = final manometer reading in mm.

a = atmospheric pressure in mm. of mercury

w = aqueous vapour pressure of water at room temperature in mm. Hg

$$\text{Then } v = \frac{760 \times 500}{(m_1 - m_2)} \left(1 - \frac{w}{a} \right)$$

MODE OF OPERATION

(a) *Filling With Hydrogen.*—Close the hydrogen delivery tube by means of a pinch clip or, if the charcoal trap is being used, close the tap thereof. Connect the exhaust outlet to vacuum and close tap F: open taps G, H and I. and evacuate the apparatus. Then attach the hydrogen inlet tube to a cylinder and sweep out the air in the safety valve and associated tubing by applying just enough pressure to blow the valve, and then allowing hydrogen to bubble through for a few seconds. If the inlet tube is detached from the cylinder without previously closing by means of a pinch clip, air will diffuse into the valve, and must be swept out again before refilling. The apparatus is then filled by opening tap F and allowing hydrogen to flow in from the cylinder until the manometer registers about 60 cm., when tap F is closed. Tap H must be open during this operation. This sequence of operations is sufficient when the apparatus is in regular use, but when first used or after being unused for some time it is advisable to sweep out the apparatus thoroughly by repeating the above sequence two or three times, depending on the vacuum available.

(b) *Hydrogenation.*—A standard joint round-bottomed flask of suitable capacity is used as reaction vessel, and an adapter is needed to connect to the hydrogen delivery tube. The adapter is made by pulling out a standard cone and joining on to a short length of 12 mm. Pyrex tubing. The joint is greased with Apiezon "L" and held together by copper wire in conjunction with two bands of brass strip, as shown in Figure 4. Estimate, or measure when maximum accuracy is required, the dead space which will exist in the flask and adapter after adding the solution to be hydrogenated. This volume is added to the appropriate reservoir volume before calculating the manometer fall, which corresponds with the theoretical volume of hydrogen to be absorbed (see below). Then check that tap G is set to the desired reservoir capacity and close tap H. Mount the reaction vessel on the shaker and connect the adapter to the hydrogen delivery tube. open the tap on the charcoal trap, if this is in circuit.

Evacuate the reaction vessel by opening tap I, and after closing it admit hydrogen from the reservoir. Whether or not this operation is repeated depends upon the vacuum available and the volatility of the solvent, although where possible it should be repeated. At this point tap H should be open: if the manometer reading is too low, let in more hydrogen from the cylinder or, when the 1 litre capacity is being used, it is only necessary to open and close tap G. Note the manometer reading, making sure that tap F is closed and tap H open; start the shaker. In order to check that hydrogen is being absorbed, it is useful to close tap H at this point in

order to obtain the magnified pressure fall referred to above. During very slow hydrogenations it is advisable to correct for any change in atmospheric pressure and room temperature that has occurred during the process and to adjust the final manometer reading accordingly.

Calculation of the Required Manometer Drop

Let t_1 = temperature (in °C.)

s = volume of the dead space (in ml.)

h = volume of hydrogen in ml. theoretically required at N.T.P.

(The other symbols are as above)

$$\text{Then } m_1 - m_2 = 760 \times \left(\frac{\frac{h(273 + t_1)}{273}}{(v + s)} \right)$$

Correction for Temperature and Pressure Variations During Hydrogenation

Let m_2 = the originally calculated final manometer reading (in mm.)

m_3 = the corrected final manometer reading (in mm.)

t_1 = initial temperature (°C.)

t_2 = final temperature (°C.)

a_1 = initial atmospheric pressure (in mm. mercury)

a_2 = final atmospheric pressure (in mm. mercury)

$$\text{Then } m_3 = \left(\frac{(273 + t_2)(m_2 + a_1)}{(273 + t_1)} \right) - a_2$$

The author wishes to express thanks to Professor W. H. Linnell for encouragement and advice during this work. Also to Dr. W. J. Arrol for suggesting the use of the charcoal trap, to Mr. P. C. Barden for constructing the shaker and to Mr. V. Askam for checking the equations.

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REVIEW ARTICLE

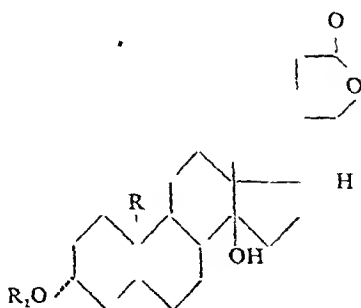
THE CHEMICAL ESTIMATION OF DIGITALIS AND STROPHANTHUS GLYCOSIDES

BY TEODOR CANBÄCK, LEG. APOT., FIL. LIC.

Director of the Pharmaceutical Control Laboratory, Stockholm, Sweden
Member of the Swedish Pharmacopœia Commission

CERTAIN glycosides of plant origin possess valuable cardiotonic properties. The Families Scrophulariaceae and Apocynaceae are the plant orders which contain most members furnishing drugs of therapeutic value. If in this connection the toad poisons are included, the heart-active substances from a chemical viewpoint may be subdivided into three large groups: the digitalis-strophanthus group, the scilla-helleborus group and the nitrogenous venoms secreted by the parotid glands of toads. The chief differences between the groups are: the members of the first group are glycosides, the aglucones of which contain a five-membered lactone ring, the members of the second group are glycosides, the aglucones of which contain a six-membered lactone ring and the members of the last group are suberylarginine derivatives of acetylated hydroxylactones more closely related to the scilla group than to the digitalis group.

Only the first group will be discussed here. The ring system of the cardiac glycosides from the genera *Digitalis* and *Strophanthus* is given below:—



The sugar moiety R₁ may be composed of as many as four sugar molecules. The sugars are for the most part α -desoxy sugars (cymarose, digitoxose and sarmentose) and digitalose, glucose and rhamnose. When an α -desoxy sugar is joined directly to the nucleus, the hydrolysis of the glycoside bond is easily effected; when rhamnose or glucose is the first sugar molecule the union is much firmer, and drastic conditions are necessary for splitting off the sugars. Digitoxin may be mentioned as an example of the first group; ouabain as an example of the second group. In fact, it is only very recently that Mannich has succeeded in

splitting off rhamnose from ouabain without dehydrating the aglucone at the same time. The aglucones are steroids* with a $\Delta\alpha:\beta$ -butenolide C_{17} side chain and they are hydroxylated in positions 3 and 14. Many of the aglucones have additional hydroxyl groups in positions 5, 12 and 16. Summarising, it may be said that the characteristic cardiac-stimulating activity of the glycosides is due to the steroid skeleton of the aglucone, the C_{17} side chain, the C_{14} hydroxyl group and to a minor extent to the structure of the sugar moiety. The glycosides are present in the plants in mixtures. From *Digitalis purpurea* a number of substances have been isolated: the purpurea glycosides, which on partial hydrolysis give digitoxin and gitoxin, gitalin, the inactive glycoside diginin, saponins such as digitonin, tannins and enzymes.

It is about fifty years since the first paper was published on the biological assay of digitalis, and there is still controversy among the biologists as to which method shall be used. In recent publications Gold *et al*¹ advocate a human assay of digitalis to overcome the discrepancy between the potencies of digitalis preparations obtained by the cat method and the actual activity in man, especially by oral administration. This discrepancy has emphasised the necessity of finding a more specific method of analysis than the crude lethal dose method. Thus it is desirable that any assay method of cardiotonic drugs should take account of not only the amount and the distribution of the active glycosides, but also of the absorbability of the active principles from the intestine.

CHEMICAL TESTING

It is symptomatic that in the last few years some very interesting papers on the chemical estimation of digitalis and strophanthus preparations have been published. Some of these papers will now be reviewed and commented upon.

The chemical assay of digitalis leaf is a complicated matter. The ideal method should give figures for:—(1) the total amount of active glycosides; (2) the amount of each different glycoside; (3) the amount of aglycones; (4) the absorbability of the glycosides. As the potency of the glycosides varies with the number of sugar molecules attached to the nucleus it is also desirable to have information of the amount of sugars attached.

The structure of the compounds also gives some clue to the solution of the problem. Purely physical methods such as ultra-violet and infra-red spectroscopy would give some information. Colorimetric methods which determine either the sugars or the butenolide group should give reliable figures of the amount of active principles present, but unfortunately no method is known, which differentiates between the glycosides and the aglucones when working on the genin part of the glycoside. However, by combination of a method based on the sugars and a method

* The alkaloids from *Erythrophleum* have cardiotonic activity. They are not steroids.

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based on the butenolide group it is easy to calculate the amounts of glycosides and aglucones present.

It should also be possible to separate the glycosides from the aglucones by chromatography on aluminium oxide, or charcoal, or paper strips or by partition chromatography. The separation of the different glycosides (or, after hydrolysis, the aglucones) would also be possible by some kind of chromatography. The chromatographic spectrum of the glycosides could be determined by the aid of pure glycosides.

The extraction of the glycosides from digitalis leaf powder or from galenicals can be done in different ways. Usually an aqueous, methyl alcoholic or ethyl alcoholic extract is prepared. The tannins are removed by shaking with fresh lead oxide or precipitated by lead acetate solution, the precipitate is filtered off and the dissolved lead removed by hydrogen sulphide or sodium phosphate. From this partially purified extract the glycosides and aglucones are extracted with chloroform. Free sugars are left in the aqueous phase. When the final estimation is made on the sugar moiety of the glycosides the latter are hydrolysed and the free sugars estimated.

This route of isolation and purification has some disadvantages, as at all stages large losses are suffered. Extraction with water or with alcohols of different strengths may result in a fractionation of the glycoside complex, as the different glycosides have very different solubilities in these solvents. When the tannins are precipitated by lead acetate, coprecipitation of the glycosides will occur, and it is difficult to wash the precipitate free from them. The extraction of the glycosides and the aglucones from the aqueous or alcoholic solution by chloroform is a very troublesome procedure. The presence of saponins enhances the solubilities of the active principles in the aqueous phase, and even repeated extraction will not give a quantitative yield.

Methylene chloride is a much better solvent than chloroform when extracting pure or purified glycosides from tablets containing lactose because the solubility of lactose in methylene chloride is much less than in chloroform. Lactose disturbs most of the known colour reactions, especially those carried out in alkaline solution, and measuring the transmission at about 500 m μ is unreliable when chloroform is used as the solvent.

METHODS DEPENDENT ON THE BUTENOLIDE SIDE CHAIN

All glycosides from the digitalis and strophanthus groups show in ethyl alcoholic solution an absorption maximum between 215 and 220 m μ in most cases at 217 m μ with log ϵ about 4.2. This absorption is very characteristic and is due to the $\Delta^{\alpha:\beta}$ -butenolide group. Of course, it is not possible to differentiate between glycosides and aglucones. The method has been used for some years in this laboratory as routine control on injections containing ouabain or digitoxin. When suitable

equipment is available and interfering substances are known to be absent, the method is very rapid.

Extinction plotted against amount of glycoside present gives a straight line when the measurements are made on the peak of the absorption curve. If alkali is added, it is possible to follow the isomerisation of the glycosides as the peak at 217 $m\mu$ gradually disappears, which is a good identification of the substance present.

The following methods depend on a reaction with the butenolide group. the Legal reaction with sodium nitroprusside and alkali, the Knudson and Dresbach method with alkaline sodium picrate solution, the Raymond method with *m*-dinitrobenzene in alkaline solution, the Kedde method with alkaline sodium 3:5-dinitrobenzoate solution and the Warren, Howland and Green method with sodium β -naphthoquinone-4-sulphonate.

The Legal reaction depends on the formation of a red colour when sodium nitroprusside is added to an alkaline solution of the glycosides. The colour test has been described by Jacobs, Hoffman and Gutus² and has been extensively used in research work on the cardiac glycosides. Qualitatively the test is performed so that a relatively large amount (0.01 g.) of the glycoside to be tested is dissolved in pyridine and an equal volume of water is added. A few drops of a 10 per cent. sodium hydroxide solution are added and then 1 ml. of 0.3 per cent. sodium nitroprusside solution. When a heart-active glycoside is present, a bright red colour develops, which slowly fades. The test has been criticised by Elderfield³, who proposed the use of potassium ferricyanide instead of sodium nitroprusside. Hardegger, Heusser and Blank⁴ have shown that the Legal test is not specific for the butenolide group, but that certain related synthetic products also give a positive reaction. The reaction probably involves a condensation of the butenolide group with the reactive NO group (in alkaline solution), and the colour is probably due to salt formation of an isonitroso derivative.

Kedde⁵ has developed a quantitative method for the assay of digitalis preparations based on the Legal reaction. He works in a buffered solution of pH 11, and obtains a relatively stable colour. The absorption band has a maximum at about 470 $m\mu$.

Some years ago Knudson and Dresbach⁶ used the Baljet⁷ reaction to estimate digitalis preparations. The Baljet reaction is based on the Jaffe reaction⁸: creatinine gives with alkaline picrate solution a red colour. The glycosides give a red-orange colour when their solutions are treated with alkaline picrate solution. The method has recently been of current interest in the United States of America and Bell and Krantz⁹ especially have contributed some interesting investigations. In a series of papers they have shown that the method gives reliable results when the transmission is measured by means of a photoelectric colorimeter. In a collaborative study¹⁰ of the assay of digitalis and its preparations by the Baljet reaction and the cat method it was shown that significant correlation between the two methods existed, showing that both methods

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measure the same activity of the drugs. Their method has been made the basis for method I, Digitoxin Colorimetric Controls, in the U.S.P. XIII. For the decolorisation of ethyl alcoholic extracts of digitalis leaf powder they use lead acetate and sodium phosphate. No extraction of the glycosides with chloroform is used, but the reagent, alkaline picrate solution, is added directly to the purified aqueous extract of the drug. After 20 minutes the difference in transmission between the extract and a blank is measured at about 525 m μ . As standards the U.S.P. digitalis reference standard tincture and the same tincture of half strength are used.

U.S.P. XIII uses absolute methyl alcohol to extract digitoxin from tablets.¹¹ It is necessary to change the composition of the reagent to avoid precipitation of digitoxin and sodium picrate in the final solution. To the methyl alcoholic digitoxin solution containing 0.1 to 1.0 mg. in 10 ml. are added 10 ml. of reagent (2 g. of trinitrophenol dissolved in methyl alcohol to 50 ml., 5 ml. of sodium hydroxide solution (1 in 10) and water to 100 ml.) and after 30 minutes the transmission is measured at about 525 m μ .

The modified Knudson-Dresbach chemical assay has been criticised from different aspects and published results indicate that the values obtained are generally higher than the values obtained by bioassay procedures. The most serious drawback is that glycosides as well as aglucones are determined and reported, as Baljet pointed out. Goldstein¹² has shown that ageing of digitalis tinctures does not apparently affect their potencies according to the chemical assay, which is rather improbable. Elmqvist and Liljestrand,¹³ working with infusions and pills, point out that while the bioassays (guinea-pig) indicate a rapid deterioration of the preparation with time, the potencies obtained by the chemical assays are constant or sometimes even increase. Vos and Welsh¹⁴ have reported similar results.

Another disadvantage of the Knudson-Dresbach method is that the difference between the absorption curve of the picrate ion in alkaline solution and the absorption curve of the test solution with alkaline picrate solution is very small, while difficulties of purely technical kinds are present. The Lambert-Beer law is not fulfilled. Lactose interferes as it gives a pronounced colour with the alkaline reagent. An advantage is that the curves obtained by plotting photometer readings against time indicate the presence of interfering substances when the resulting curves differ in shape from those obtained with a standard.

In 1935, Marthoud¹⁵ pointed out that a solution of *m*-dinitrobenzene and heart-active glycosides in ethyl alcohol, on addition of alkali, develops a bright blue-violet colour. This colour test was quantitatively used by Raymond¹⁶ to estimate the amount of ouabain in East African arrow poisons prepared from seeds of *Strophanthus* species and from *Acocanthera* wood. Rasmussen¹⁷ used the method to estimate ouabain in injections. Anderson and Chen¹⁸ to assay digitoxin. Canbäck modified the method and estimated digitalis, strophanthus¹⁹ and uzara

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preparations²⁰. The reaction is a special case of the general reaction of *m*-dinitrobenzene with active methylene groups in alkaline solution.

Anderson and Chen¹⁸ use the following technique: 150 to 250 μ g. of digitoxin is dissolved in 10 ml. of 47.5 per cent. ethyl alcohol and 1 ml. of a 1 per cent. *m*-dinitrobenzene solution in absolute ethyl alcohol is added. The mixture is placed in an ice-bath for 5 to 10 minutes. Then 2 ml. of a 20 per cent. solution of sodium hydroxide is added and the mixture returned to the ice-bath. Exactly 5 minutes after the addition of the sodium hydroxide the transmission is measured in a photoelectric colorimeter equipped with an orange filter. Water is used as a blank. The amount of digitoxin present is read from a standard curve. The colour fades fairly rapidly and it is necessary to read exactly 5 minutes after the addition of the alkali. The colour intensity is dependent on the alcoholic strength and the temperature, both of which must be rigidly controlled.

To overcome most of the difficulties the following technique¹⁹ for the estimation of digitoxin tablets has been used in this laboratory:—

As much of well powdered tablets as corresponds to 800 μ g. of digitoxin is weighed into a 15-ml. centrifuge tube and 10.00 ml. of methylene chloride is added. The tube is immediately closed with a cork covered with tinfoil and shaken in a machine for 30 minutes. Then the mixture is centrifuged at 2,000 r.p.m. for some minutes. 5.00 ml. of the clear solution is transferred to a beaker and the methylene chloride evaporated in a vacuum at 50°C. The residue containing the glycoside and any lubricant that may be present (stearic acid), is treated with 5 ml. of 50 per cent. ethyl alcohol and carefully warmed to about 70°C. for a few seconds. This warming is necessary to dissolve the glycosides under the prescribed conditions. The solution is cooled and the separated stearic acid removed by filtration. The filtrate is adjusted to 6.00 ml. with 50 per cent. ethyl alcohol. To this solution 2.00 ml. of 10 N sodium hydroxide solution is added. At the same moment as the alkali is added a stop watch is started and the extinction is determined at about 620 $m\mu$ every 30 seconds. The logarithms of the extinctions were plotted against time and a straight line is obtained. Log extinction at zero time is read from the graph. A standard curve is prepared from "zero" extinctions, obtained with weighed amounts (200 to 600 μ g.) of digitoxin.

In this modification the Raymond method is unaffected by reasonable variations in alcoholic strength of the final solution and by temperature variations within the range 17° to 23°C. From measurements made on digitoxin, cymarín, strophanthin, ouabain, gitoxin, digitoxigenin, strophanthidin, and on the synthetic analogue β -phenyl- $\Delta^{\alpha:\beta}$ -butenolide the extrapolated molar "zero" extinction coefficient has been found to be about 12,000 or $\log \epsilon = 4.08$ at 620 $m\mu$. Lactose interferes, therefore it is preferable to extract digitoxin with methylene chloride, instead of with chloroform, from tablets containing this sugar. Injections, even those containing glycerol, are diluted with ethyl alcohol and directly estimated.

The Raymond method has the following advantages over the Knudson-Dresbach method. The absorption maximum lies far in the

red part of spectrum at 620 $m\mu$, and thus yellow and red colours from impurities have a less serious effect on the measurements than in the Knudson-Dresbach method. The reagent is colourless and stable for some days. The measurements are made very rapidly. Another positive character is that the glycosides from *Scilla* and *Helleborus* do not give colours. A disadvantage is that it is necessary to work with speed in order to obtain sufficient points to construct the curve. The Raymond method, like the Knudson-Dresbach method, makes no distinction between glycosides and aglucones.

The Raymond method has been discussed more in detail than the other methods because in my opinion this method is the most promising of those acting on the lactone side chain. In combination with one of the methods discussed below acting on the sugar moiety, it seems possible to devise a good chemical assay of digitalis preparations.

A new reagent for digitalis glycosides has recently been proposed by Kedde⁵. He uses 3:5-dinitrobenzoic acid in alkaline solution to estimate the sum of glycosides and aglucones. The mechanism of the reaction is probably the same as in the Raymond reaction.

Another new reagent is sodium β -naphthoquinone-4-sulphonate used by Warren, Howland and Green²¹ for the estimation of digitoxin. Ehrlich and Herter²² and Feigl and Frehden^{23,24} described the use of the reagent for the detection of active methylene groups. The colour produced is probably due to the formation of a quinoid condensation product. The condensation reaction is carried out in alkaline solution and the colour produced is purple. On acidification with acetic acid a stable yellow colour is obtained which may be extracted by dibutyl phthalate.

Warren, Howland and Green²¹ assay digitoxin tablets in the following way. Tablets are ground to a fine powder, which is extracted with boiling chloroform. After filtering, the volume is adjusted and an aliquot part, corresponding to 200 μ g. of digitoxin, is evaporated in an Erlenmeyer flask on a steam bath. 0.1 ml. of chloroform is added and the flask is shaken to wet all the residue. Then 4 ml. of alcohol and 0.5 ml. of 0.05 N sodium hydroxide solution are added. The flask is placed in a 100°C. bath and after 1 minute 1 ml. of the reagent (containing 0.024 per cent. of sodium β -naphthoquinone-4-sulphonate and 0.024 per cent. of sodium sulphite in distilled water) is rapidly added. One and one-half minutes after the addition of the reagent 0.5 ml. of acetic acid solution (containing 13 per cent. of acetic acid in alcohol) is added with agitation. The flask is removed from the bath, and cooled under the tap. The volume is adjusted to 25 ml. with alcohol and the transmission measured within 2 hours at about 450 $m\mu$. A blank is prepared in the same manner omitting the digitoxin. As aldehydes give a positive reaction with the reagent, aldehyde-free ethyl alcohol must be used.

As expected, the authors report that lanatoside A (digitoxin derivative) and lanatoside C (digoxin derivative) gave a strong positive reaction. Curiously, however, gitoxin and its derivative lanatoside B gave only a weakly positive test. The only structural difference between digitoxin

and gitoxin is the additional hydroxyl group on C_{16} , and it is hard to believe that this hydroxyl group could interfere. The slight solubility of gitoxin may be responsible for the different behaviour of the glycoside. Digitonin gave a negative reaction.

METHODS DEPENDENT ON THE SUGAR MOIETY OF THE GLYCOSIDES

Many methods for the detection and determination of the sugars in the heart-active glycosides have been reported. All the digitalis glycosides of interest are built up according to the same scheme:—aglucone + 3 digitoxose (+ glucose). The strophanthus glycosides contain other sugars instead of digitoxose, for instance, ouabain contains rhamnose, strophanthin-k β -cymarose and glucose, and cymaridin, cymarose.

On the basis of a sugar determination it is for the moment a technically easier task to devise an assay method which is specific for the glycosides and excludes the aglucones, than to construct one on the basis of a determination of the butenolide side chain. In the first case it is only necessary to extract the glycosides and the aglucones from an aqueous or ethyl alcoholic extract and to determine the sugars which are set free in a separate hydrolytic reaction. In the latter case it is necessary to separate glycosides and aglucones by a tedious process and then to estimate the glycosides. When working with a sugar method it is, however, always a good thing to remember that the part of the molecule actually measured is not the specific one. On principle those methods which depend on a reaction with the butenolide side chain should therefore be preferred.

Of the proposed colorimetric sugar methods only two recently described modifications of the Keller-Kiliani test will be discussed. The Keller-Kiliani test depends on the colours obtained when concentrated sulphuric acid is added to digitoxose dissolved in glacial acetic acid containing traces of ferric chloride. As early as 1906 Cloetta and Fischer²⁵ tried this method for the estimation of heart-active glycosides.

Recently James, Laquer and McIntyre²⁶ have described a modification that has been introduced in the U.S.P. XIII as Method II, Digitoxin Colorimetric Controls. They assayed digitoxin tablets in the following way. Ground tablets corresponding to 4 mg. of digitoxin were extracted overnight with 50 ml. of chloroform. The supernatant chloroform was filtered and the residue extracted 6 times with 5-ml. quantities of chloroform. The volume was adjusted and an aliquot part of the chloroform solution corresponding to 200 μ g. of digitoxin was evaporated to dryness. To the residue were added 3 ml. of glacial acetic acid, 0.10 ml. of 5 per cent. ferric chloride solution ($FeCl_3 \cdot 6H_2O$) and 0.25 ml. of concentrated sulphuric acid. The transmission was measured at 15-minute intervals until a maximum reading was reached, usually within 15 to 45 minutes. The light filter had a maximum transmission between 500 and 570 $m\mu$. The Lambert-Beer law is fulfilled.

In assaying injections containing glycerol it is necessary to prepare a

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special graph from measurements made on solutions containing known amounts of glycerol and digitoxin.

In a comprehensive study Soos²⁷ has investigated the details of the Keller-Kiliani test when applied to digitalis leaf. He used the reagent recommended by Lindewald^{28,29}, which consists of 97 ml. of glacial acetic acid, 2 ml. of concentrated sulphuric acid and 1 ml. of a 5 per cent. ferric chloride aqueous solution. Soos assays digitalis leaf in the following way. 1.50 g. of powdered leaf is moistened with a small quantity of water in a mortar and allowed to swell during 15 minutes. Then the mixture is washed into a flask with water and the weight adjusted to 151.5 g. The mixture is shaken now and then during 1 hour. After that 15 ml. of a 15 per cent. lead acetate solution is added. The precipitate is allowed to settle and the clear supernatant solution is filtered. 110 g. of the filtered solution, corresponding to 1.00 g. of the drug, is extracted with 3×25 ml. of chloroform. The chloroform solution is dried with sodium sulphate and filtered into an Erlenmeyer flask. The chloroform is distilled off and the residue dissolved in 10 ml. of freshly prepared reagent. The extinction is measured at 30-minute intervals and the maximum value recorded during 5 hours. As the reaction is sensitive to light it is preferable to let the solution stand in the dark during this time. The absorption curve has a maximum at 570 m μ .

Soos points out that unknown impurities in the glacial acetic acid he used had a very pronounced effect on the development of the colour. Thus it is necessary to construct a graph with the glacial acetic acid actually used. As standard substances either digitoxin or digitoxose may be used. Drawbacks of the methods are that the development of the colour takes 2 to 5 hours and that the extraction of the glycosides from the aqueous extract with chloroform is never quantitative. Usually about 80 per cent. is recovered. The presence of saponins in the drug decreases the yield obtained by extraction. Nevertheless, the method probably is the best of the known methods for assaying digitalis leaf. It is the only method that measures the glycosides present in the drug excluding the aglucones. But, as pointed out previously, the method has the defect of not measuring an essential part of the heart-active molecule.

The time seems to be near when the pharmacopœias will have seriously to discuss the problem of the assay of drugs containing heart-active glycosides in the light of the experience gained with chemical methods. A combination of a method assaying the total amount of glycosides and aglucones present, and a method assaying only glycosides would appear to define most of the necessary characteristics of the drugs.

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RESEARCH PAPERS

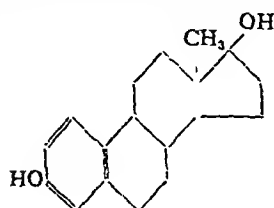
SYNTHETIC ŒSTROGENS

BY EDWARD R. CLARK AND W. H. LINNELL

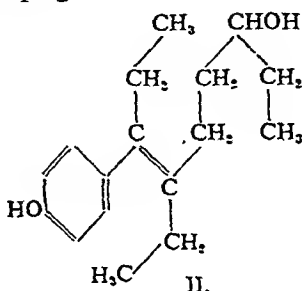
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It is remarkable that all the most active synthetic Œstrogens, with the exception of the Œstrogenic carboxylic acids of the doisyonic acid type, possess two phenolic hydroxyl groupings, and in this respect differ from the potent natural Œstrogen, Œstradiol (I), which has one phenolic and one alcoholic hydroxyl. It was considered of interest, therefore, to synthesise the compound II in an attempt to obtain a synthetic Œstrogen resembling, more closely, the Œstradiol structure with regard to the chemical nature of the functional groupings.

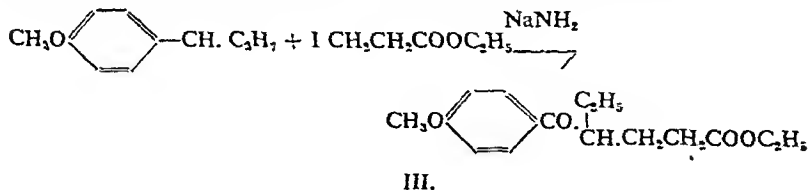


I.



II.

Attempts to synthesise 4-ethyl-5-(*p*-anisyl)- $\Delta^{4,5}$ -heptenoic acid as an intermediate in the production of II were unsuccessful. Ethyl 4-(*p*-methoxybenzoyl)-caproate (III) was obtained from ethyl β -iodopropionate¹ and the sodium enolate of *p*-methoxybutyropheneone².



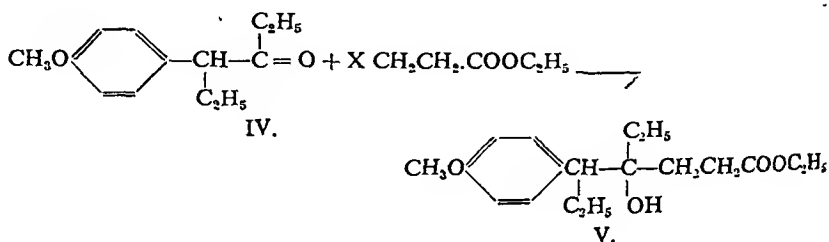
Treatment of III with one molar equivalent of ethyl magnesium iodide, or ethyl lithium, did not yield the anticipated hydroxyester. Similarly treatment of 4-(*p*-methoxybenzoyl)-caproic acid with three equivalents of ethyl magnesium iodide gave a theoretical recovery of the keto-acid.

An attempt to condense ethyl α -(*p*-anisyl)-butyrate with diethyl succinate using sodium as the condensing agent yielded succinyl succinate as the sole product. Similar results were obtained in the attempted condensation of ethyl α -phenylbutyrate with succinic ester using alcohol-free sodium ethoxide and α -phenylbutyryl chloride with succinic ester using triphenylmethyl sodium.

The successful reaction between 6-methoxytetralone and ethyl β -bromo-

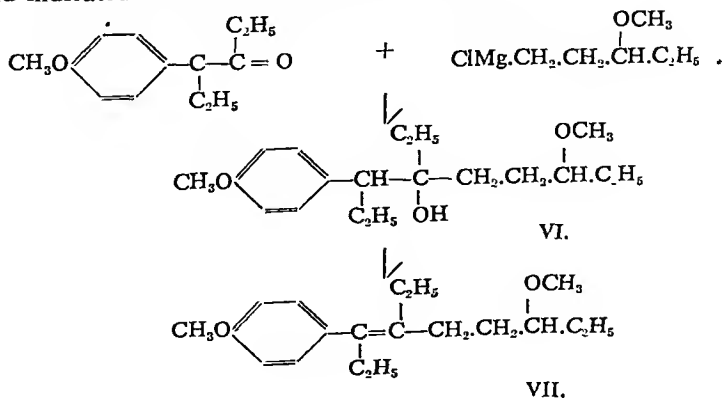
propionate using magnesium in toluene to produce β -(6-methoxy-3:4-dihydronaphthyl-(1))-propionic acid³, suggested that a similar reaction using 4-(*p*-anisyl)-3-hexanone (IV) in place of 6-methoxytetralone would yield the required intermediate.

The 4-(*p*-anisyl)-3-hexanone required was obtained by the action of diethyl cadmium on α -(*p*-anisyl)-butyryl chloride, the product being found to be identical with that formed by the action of ethyl iodide on *p*-methoxybenzyl-ethyl-ketone in the presence of alcohol-free sodium ethoxide⁴. On refluxing ethyl β -bromopropionate⁵, magnesium and 4-(*p*-anisyl)-3-hexanone in toluene only a very poor yield of the product V was obtained. Substituting ethyl β -iodopropionate for the bromo analogue and conducting the experiment in ether gave a similar poor yield of V.



An attempt to remove the elements of water from this by heating on a water-bath with 2 per cent. of iodine for 1 hour was unsuccessful.

The preparation of the Grignard complex from γ -methoxyamyl chloride⁶ proved difficult, 6 hours' refluxing with activated magnesium in ether being required for completion of the reaction. Addition of 4-(*p*-anisyl)-3-hexanone to 2 equivalents of this Grignard complex gave, on working up in the normal way, a 67 per cent. yield of 3-(*p*-anisyl)-4-ethyl-7-methoxy-4-nonanol (VI). Dehydration by heating on a water-bath for 1 hour with 1 per cent. of iodine yielded an ethylenic compound. Ozonolysis of this compound gave an 88 per cent. yield of *p*-methoxypropiophenone, identified as its 2:4-dinitrophenylhydrazone and indicated VII as the structure of the olefine.

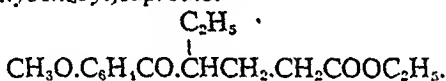


Demethylation of VII was achieved by heating with hydrogen bromide and acetic acid in an atmosphere of carbon dioxide for 2 hours. Purification of the product by adsorption chromatography on alumina yielded a viscous brown oil which contained no methoxyl and two active hydrogens. Carbon and hydrogen analysis agreed with that calculated for 3(*p*-hydroxyphenyl)-4-ethyl-7-hydroxy- $\Delta^{3,4}$ -nonene (II). The failure to isolate the two possible geometrical isomers on chromatographic adsorption of II suggests that the product consisted of only one isomer. The use of iodine for the formation of the double bond and hydrogen bromide for demethylation of VII supplied conditions suitable for the interconversion of geometrical isomers and would be expected to produce the more stable form which in most cases of *cis-trans* pairs has the *trans*-configuration. It appears likely, therefore, that the compound II possesses the *trans* structure.

We are indebted to Professor Buttle and Dr. F. J. Dyer, of the Pharmacological Department of this School for the biological examination of compounds VII and II. The dimethyl ether VII was found to be inactive on administration in arachis oil solution of 20 mg. per 20 g. mouse. The phenolic alcohol II, in propylene glycol solution, was found to possess less than 1/40,000 of the activity of stilbœstrol.

EXPERIMENTAL

Ethyl 4-(*p*-methoxybenzoyl)caproate.



The sodium enolate of *p*-methoxybutyrophenone was prepared by adding 50 g. of the ketone, dissolved in 50 ml. of dry benzene, in 10 aliquot parts at 10 minute intervals, to 12 g. of finely powdered sodamide in 250 ml. of dry benzene, the mixture being refluxed vigorously until no further ammonia was evolved. To the cooled benzene solution was added gradually, with rapid stirring, a solution of 55.6 g. of ethyl β -iodopropionate in 30 ml. of dry benzene, the reaction flask being kept cold in an ice bath. After completion of the addition, stirring was continued for 1 hour at room temperature and then with refluxing for 30 minutes. The cooled product was poured into 300 ml. of water, the benzene layer was separated and the aqueous layer extracted twice with benzene. The combined benzene solution and extracts were washed with sodium thiosulphate solution and finally dried over anhydrous sodium sulphate. Evaporation of the benzene and distillation of the residue *in vacuo* yielded a golden yellow oil, b.pt. 180° to 182°C./1.2 mm. Yield, 35 to 40 per cent. of theoretical. Found: Eq. Wt. 273; $\text{C}_{16}\text{H}_{22}\text{O}_4$ requires 278.

4-(*p*-methoxybenzoyl)caproic acid. $\text{CH}_3\text{OC}_6\text{H}_4\text{CO} \cdot \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$.

10 g. of the ethyl ester were refluxed with 100 ml. of N alcoholic potash for 3 hours. The cooled solution was diluted to 500 ml. with water and acidified with hydrochloric acid. The acid was filtered off and recrystallised from 40 per cent. aqueous alcohol, yielding 8.5 g. (94.5

per cent. of theory) of creamy white crystals, m.pt. 83.5° to 84°C . Found: C, 66.5; H, 6.86; CH_3O , 10.9 per cent., Eq. Wt. 250.8; $\text{C}_{14}\text{H}_{18}\text{O}_2$ requires C, 67.2; H, 7.2; CH_3O , 12.4 per cent., Eq. Wt. 250.

Diethyl p-methoxyphenylethylmalonate.

23.3 g. of sodium was added to 330 g. of absolute alcohol. After the solution had cooled to about 60°C . a mixture of 150 g. of ethyl oxalate and 194 g. of ethyl *p*-methoxyphenyl acetate⁷ was added quickly with rapid stirring. The temperature, which dropped to about 40°C ., was brought back to 60°C . and held at that temperature until the sodium salt of *p*-methoxyphenyl oxalacetic ester separated thickly. This occurred in about 2 or 3 minutes. The mixture was held at 60°C . for a further 30 minutes, after which the sodium salt was acidified with 70 g. of concentrated sulphuric acid diluted with 200 ml. of water. The mixture formed two layers. After verifying that the aqueous layer was acid to congo-red, the ester was taken into benzene, care being taken to avoid the crystallisation of sodium sulphate decahydrate by keeping the temperature above its melting-point. The benzene was distilled under reduced pressure. The distillation flask was then fitted with a still head carrying a thermometer dipping beneath the surface of the liquid, and an air condenser. The ester was heated on an oil bath, raising the temperature to 190°C . in 30 minutes and held at that temperature for a further hour. The crude *p*-methoxyphenylmalonic ester was then fractionated under reduced pressure yielding 235 g. (86.5 per cent. of theoretical) of a yellow oil. B.pt. 159° to $161^{\circ}\text{C}/2\text{ mm. Hg}$.

Diethyl p-methoxyphenylethylmalonate. $\text{CH}_3\text{OC}_6\text{H}_4\text{C}(\text{C}_2\text{H}_5)(\text{COOC}_2\text{H}_5)_2$.

14.6 g. of sodium was dissolved in 175 ml. of absolute alcohol and to the solution of sodium ethoxide so formed was added, at 50° to 60°C ., 180 g. of diethyl *p*-methoxyphenylmalonate. When the temperature had fallen to about 35°C ., 103.75 g. of ethyl iodide were added as rapidly as possible and the temperature held at 35°C . for 6 hours with stirring. The alcohol was then removed by distillation, the residue diluted with 250 ml. of water and made acid to methyl red with acetic acid. A little sodium sulphite was added to remove any free iodine and the ester taken into benzene. After removal of the solvent the ester was distilled under reduced pressure, yielding 164 g. of a yellow oil with a boiling point identical with the starting material. Yield, 82.5 per cent. of theoretical. Eq. Wt. found 147.7; $\text{C}_{15}\text{H}_{22}\text{O}$ requires 147.

*α -(*p*-Anisyl)-butyric acid.* $\text{CH}_3\text{OC}_6\text{H}_4\text{CH}(\text{C}_2\text{H}_5)\text{COOH}$.

100 g. of *p*-methoxyphenylethylmalonic ester were saponified by refluxing on a water-bath with 750 ml. of 10 per cent. alcoholic potash for 3 hours. The cooled solution was diluted with 3 l. of water and acidified with hydrochloric acid and the precipitated acid filtered off at the pump. Evaporation of the filtrate to about half its volume gave, on cooling, a fourth crop of crystals. Recrystallisation from light petroleum (40° to 60°C .) yielded 63 g. of needle-shaped crystals, m.pt. 64° to 65°C . Yield, 92 per cent. of theoretical. Found: C, 68.5; H, 7.3; CH_3O , 14.9 per cent., Eq. Wt. 193. $\text{C}_{11}\text{H}_{14}\text{O}_3$ requires C, 68.8; H, 7.2; CH_3O , 16.0 per cent., Eq. Wt. 194.

α-(*p*-Anisyl)-butyryl chloride. $\text{CH}_3\text{OC}_6\text{H}_4\text{CH}(\text{C}_2\text{H}_5)\text{COCl}$.

39 g. of *α*-(*p*-anisyl)-butyric acid was refluxed with 70 g. of thionyl chloride for 3 hours. The excess of thionyl chloride was distilled off and the residue distilled under reduced pressure, yielding a pale yellow pungent liquid. B.pt. $120^\circ\text{C}/4$ mm. Hg. pressure. Yield 40 g., 94 per cent. of theoretical.

4-(*p*-Anisyl)-3-hexanone. $\text{CH}_3\text{O.C}_6\text{H}_4\text{CH}(\text{C}_2\text{H}_5).\text{CO.C}_2\text{H}_5$.

Ethyl magnesium bromide was prepared from 13.6 g. of ethyl bromide and 3 g. of magnesium in 100 ml. of absolute ether. The solution was cooled in an ice bath and 12 g. of dry, finely powdered cadmium chloride added. After refluxing for 45 minutes the mixture gave no colour with Michler's ketone indicating the absence of any Grignard complex. The reflux was replaced by a condenser set for distillation and nearly all the ether removed by heating on a water-bath. 50 ml. of dry benzene was then added and 20 ml. distilled. A further 50 ml. of dry benzene was then added to the mixture. The reflux condenser was replaced and to the cooled benzene solution of diethyl cadmium, was added, with stirring, a solution of 21.3 gm. of *α*-(*p*-anisyl)-butyryl chloride in 20 ml. of benzene, during about 3 minutes. The mixture was stirred at room temperature for 15 minutes and then at 40°C . for 2 hours. The cooled reaction mixture was decomposed by pouring on to 150 g. of ice and 70 ml. of 2N sulphuric acid. The benzene layer was separated and the lower aqueous layer shaken with three further quantities of benzene. The combined benzene solution and extracts were washed with dilute sodium carbonate solution and finally dried over anhydrous sodium sulphate. The benzene was removed under reduced pressure and the crude ketone fractionated, yielding a colourless oil boiling at 132° to $134^\circ\text{C}/2.5$ mm. Hg. pressure. Yield, 71 per cent. of theoretical.

Semicarbazone: m.pt. 131.5° to 132°C . Found: C, 64; H, 8.05; N, 15.4 per cent.; $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}$ requires C, 63.8; H, 7.96; N, 15.9 per cent.

2:4-Dinitrophenylhydrazone: m.pt. 91.5 to 92°C . Found: C, 58.8; H, 5.7; N, 14.8 per cent.; $\text{C}_{18}\text{H}_{23}\text{N}_5\text{O}_5$ requires C, 59.25; H, 5.45; N, 14.5 per cent.

3-(*p*-Anisyl)-4-methyl- $\Delta^{3,4}$ -hexene, $\text{CH}_3\text{O.C}_6\text{H}_4.\text{C}(\text{C}_2\text{H}_5):\text{C}(\text{CH}_3).\text{C}_2\text{H}_5$.

5.15 g. of 4-(*p*-anisyl)-3-hexanone, dissolved in 20 ml. of absolute ether, was added during 15 minutes, with cooling and stirring, to 1.5 equivalents of methyl magnesium iodide in 30 ml. of ether. The mixture was then refluxed for 3 hours, cooled, and then decomposed by pouring on to 50 g. of ice and 35 ml. of dilute sulphuric acid. The ethereal layer was separated and the aqueous portion extracted thoroughly with ether. The combined ethereal solution and extracts were washed with sodium thiosulphate solution and dried over anhydrous sodium sulphate. Distillation yielded a colourless oil boiling at 99° to $105^\circ\text{C}/0.8$ mm. Hg. pressure. Yield 3.38 g.

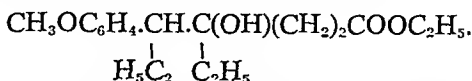
Distillation, under reduced pressure, of this oil with 2 per cent. of iodine and purification of the distillate in the normal manner yielded a colourless oil distilling at a bath temperature of 95° to 100°C . and 0.05

mm. Hg. pressure. Found C, 81.56; H, 9.3; CH_3O , 15.3 per cent.; $\text{C}_{14}\text{H}_{20}\text{O}$ requires C, 82.3; H, 9.8; CH_3O , 15.2 per cent.

The compound contained no active hydrogen.

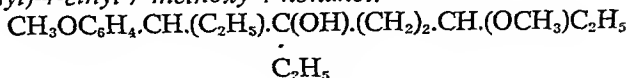
Demethylation of 3-(p-Anisyl)-4-methyl- $\Delta^{3,4}$ -hexene.

1.5 g. of the above methoxy compound was heated with three equivalents of methyl magnesium iodide under a reflux condenser, the temperature being raised slowly to 165°C . and then maintained at this temperature for 3 hours. The cooled reaction mixture was then decomposed with ice and dilute sulphuric acid, the ethereal layer separated and the aqueous layer extracted with ether. The combined ethereal solution and extracts were washed with sulphate solution and then extracted with dilute aqueous sodium hydroxide. Acidification of the alkaline extract with dilute sulphuric acid, extraction with ether and distillation yielded a yellow glass distilling at 108° to 118°C . / 0.8 mm. Hg. pressure. *Ethyl 4-ethyl-5-(p-anisyl)-4-hydroxyheptanoate.*



- A. 5.15 g. of 4-(p-anisyl)-3-hexanone was refluxed with 0.61 g. of magnesium and 4.43 g. of ethyl β -bromopropionate in 50 ml. of dry toluene. Addition of 0.3 g. of iodine started the reaction. After 3 hours refluxing, the mixture was cooled and decomposed with ice and dilute sulphuric acid, the toluene layer separated and the aqueous layer extracted with ether. The combined toluene solution and ethereal extracts were washed with dilute sodium thiosulphate solution and finally dried over anhydrous sodium sulphate. Distillation yielded 0.38 g. of a light yellow, semi-solid substance boiling at 145° to 155°C . / 0.3 mm. Hg. pressure.
- B. As method A, except for the use of ethyl β -iodopropionate in place of the β -bromo analogue and ether instead of toluene. 6 hours refluxing was required for solution of the magnesium. On work-up, as before, there was obtained a yellow semi-solid fraction distilling at 115° to 120°C . / 0.05 mm. Hg., which proved to be the same as that obtained in A. Yield, 0.55 g. Found: C, 69.7; H, 8.7; CH_3O , 10.7 per cent.; $\text{C}_{18}\text{H}_{25}\text{O}_4$ requires C, 70.1; H, 9.08; CH_3O , 10.05 per cent.

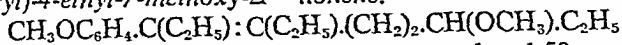
3-(p-Anisyl)-4-ethyl-7-methoxy-4-nonanol.



1.2 g. of magnesium turnings, 30 ml. of dry ether and 6.8 g. of freshly distilled γ -methoxyamyl chloride were refluxed together, with stirring. Two or 3 drops of methyl iodide helped to initiate the reaction which was very slow, requiring 6 hours for completion. To the cooled ethereal solution of γ -methoxyamyl magnesium chloride was added, during 30 minutes, 5.15 g. of 4-(p-anisyl)-3-hexanone dissolved in 10 ml. of dry ether. Stirring was continued and the mixture refluxed for 4 hours. Decomposition of the complex with ice and dilute sulphuric acid, yielded,

on working up, 5.15 g. of a viscous yellow oil boiling at 140° to $145^{\circ}\text{C.}/0.04$ mm. Hg. pressure. Found: C, 74.3; H, 10.38; CH_3O , 20.06 per cent.; $\text{C}_{19}\text{H}_{32}\text{O}_2$ requires C, 74.02; H, 10.39; CH_3O , 20.13 per cent.

3-(p-Anisyl)-4-ethyl-7-methoxy- $\Delta^{3,4}$ -nonene.



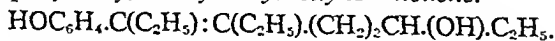
4.5 g. of 3-(*p*-anisyl)-4-ethyl-7-methoxy-4-nonenol and 50 mg. of iodine were heated together on a water-bath for 1 hour under a reflux air condenser. The resultant dark brown oil was dissolved in ether, washed with sodium thiosulphate solution and the ethereal solution then dried over anhydrous sodium sulphate. Distillation yielded a light yellow oil, boiling at 130° to $135^{\circ}\text{C.}/0.1$ mm. Hg. pressure. Found: C, 78.08; H, 10.08; CH_3O , 21.48 per cent.; $\text{C}_{19}\text{H}_{30}\text{O}_2$ requires C, 76.63; H, 10.34; CH_3O , 21.38 per cent.

Ozonised oxygen was passed into a solution of 0.5 g. of the above ethylenic compound in 50 ml. of chloroform for the calculated time. The chloroform was then removed under reduced pressure and the ozonide decomposed with iced water. Extraction with ether and evaporation of the solvent yielded a semi-solid residue. This was dissolved in light petroleum (40° to 50°C.) and passed through a column of activated alumina. The chromatogram showed three zones, (1) a narrow dark band at the top, (2) a broad pale-yellow zone, (3) a narrow band exhibiting a blue fluorescence under ultra-violet light, near the bottom. Washing with light petroleum removed the lowest band, but continued washing did not appear to remove the yellow zone. Elutriation with benzene and ether removed the yellow zone leaving a small dark brown band at the surface. Evaporation of the solvents yielded:

- 0.14 g. of a white wax-like substance which was insoluble in methyl and ethyl alcohols and water.
- 20 mg. of an oil which gave a red oil on addition of a saturated alcoholic solution of 2:4-dinitrophenylhydrazine sulphate. Crystallisation of this oil could not be brought about.
- 0.25 g. of an oil which yielded a 2:4-dinitrophenylhydrazine melting at 185° to 187°C.

Mixed melting-point with a sample of *p*-methoxypropiophenone 2:4-dinitrophenylhydrazine, 186° to 187°C.

3-(p-Hydroxy)-4-ethyl-7-hydroxy- $\Delta^{3,4}$ -nonene.



0.5 g. of 3-(*p*-anisyl)-4-ethyl-7-methoxy- $\Delta^{3,4}$ nonene was heated in an oil bath at 140°C. for 2 hours, with 1.5 ml. of 47 per cent. hydrobromic acid and 6 ml. of glacial acetic acid in an atmosphere of carbon dioxide. The reaction mixture was then cooled, diluted with water and made just alkaline with sodium hydroxide solution, keeping the mixture cold in an ice bath. The brown semi-solid product was taken into ether, the ethereal solution washed with water and finally dried. On distillation, 0.4 g. of a viscous brown oil was obtained.

A solution of this in 40 ml. of cyclohexane was passed through a column of activated alumina. Development of the chromatogram with

benzene revealed a narrow dark brown band at the top of the column, below this a broad yellow zone and at the bottom a narrow band exhibiting a blue fluorescence under ultra-violet light. Continued elutriation with benzene removed the blue-fluorescent band. A mixture of equal parts of benzene and acetone removed the yellow zone.

Distillation of this latter elutriate yielded 270 mg. of a very viscous oil which could not be crystallised. Found: C, 77.94; H, 9.91; CH_3O , 0 per cent.; $\text{C}_{17}\text{H}_{16}\text{O}_2$ requires C, 77.86; H, 9.92; CH_3O , 0 per cent.

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THE PHARMACOLOGICAL ACTIONS OF *ERYTHRÆA SPICATA*

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Received September, 1948

ERYTHRÆA SPICATA is an annual herb belonging to the family Gentianaceæ. It grows wild in damp places specially in rice fields. Muschler¹ described this plant and other species growing in Egypt, where the public has used extracts as an antidote for scorpion sting, but one of us² has shown that it has no practical effect for this purpose. Gaston Bonnier³ (1886) examined the European species *Erythræa Centaurium*. He found that it contains two glycosides and stated that it was antipyretic. Washburn⁴ stated that it contained a bitter principle similar to that in gentian and stated that it was a diaphoretic and febrifuge. Gathercoal⁵ said that it contained a glycoside, erythrocentaurin, and a resin.

Recently, attention was drawn to *Erythræa spicata* as it is believed to be efficacious in hypertension. The present work was therefore undertaken.

EXPERIMENTAL

Preparation of solutions.—An alcoholic extract of the plant was concentrated to a semi-solid consistency so that 0.3 g. of concentrated extract corresponded to 1 g. of the powdered plant. This concentrated extract (alcohol free) was dissolved in Ringer's or Locke's solution to the required concentration and the pH of the solution was adjusted before the experiment to pH 7.6.

Toad's Heart.—The method used was a modification of Syme's method of perfusion. The drug produced depression in the force of

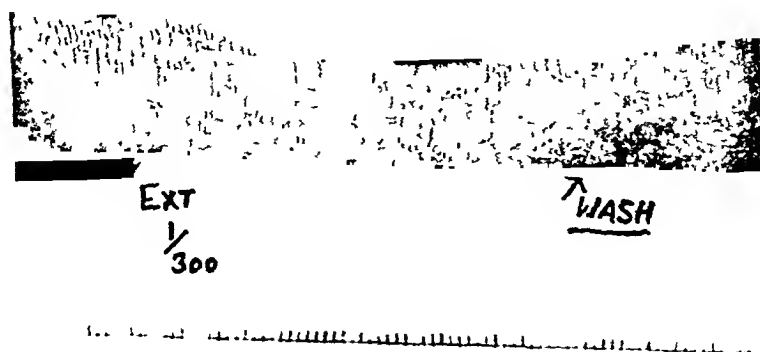


FIG 1. Effect of extract of *Erythræa spicata* extract (1 in 300) on toad's heart

contraction of the heart (Fig. 1). The lowest concentration which just produced depression of the heart was 1 in 600 of the extract. The same effect was produced by the drug after atropinising the heart.

Rabbit's Heart.—A modification of Gunn's method was used. No change in the amplitude or rate of the heart was demonstrated by 1 in 1000 but there was an increase in the coronary outflow varying from 30 to 40 per cent.

Blood Vessels.—Trendelenberg's method was used for perfusion of toad's vessels. The drug produced definite dilatation of blood vessels in dilutions between 1 in 100 and 1 in 5000 of the extracts. Solutions of 1 in 100 produced an increase of the flow varying from 68 per cent. to 100 per cent. averaging 86 per cent. and 1 in 1000 produced an increase varying from 39 per cent. to 73 per cent. with an average of 58 per cent. and 1 in 5000 produced an average increase of 19 per cent.

Intestine.—A modified Magnus technique was employed. The drug produced definite relaxation of the intestine of the rabbit (Fig. 2).

Dilutions of 1 in 1000 produced in some cases very strong inhibition of the contractions, while 1 in 10,000 produced just noticeable depression.



FIG. 2. Effect of *Erythræa spicata* extract (1 in 4500) on rabbit's intestine.

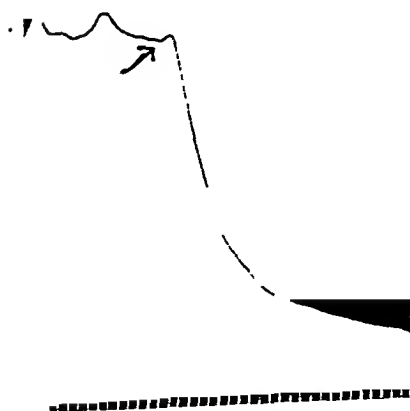


FIG. 3. Effect of *Erythræa spicata* extract (1 in 1000) on guinea-pig's uterus.

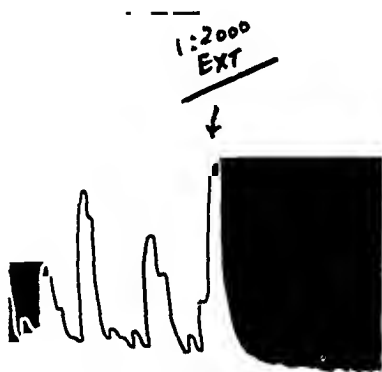


FIG. 4. Effect of *Erythræa spicata* extract (1 in 2000) on rabbit's uterus.

Uterus.—The drug produced definite relaxation of the rabbit's and guinea-pig's uteri whether pregnant or not, in all dilutions tested which ranged from 1 in 8000 to 1 in 1000 (Figures 3 and 4).

Action on Blood Pressure.—Dogs anaesthetised by chloralose intravenously after preliminary ether anaesthesia were used. The blood pressure was recorded from the carotid artery by mercury manometer on smoked paper in the ordinary way. The drug was injected intravenously through a cannula into the jugular vein. The drug produced a definite fall of blood pressure; the greater the dose injected, the greater the fall of blood pressure and the longer the duration of the fall. Experimental results in two animals may be quoted. (a) In a dog of 15 kg. body weight injected with 0.3 g. of extract, the blood pressure reading was reduced from 185 mm. Hg to 155; 1.5 g. reduced it to 115 mm. Hg and 3 g. to 95 mm. Hg.

(b) In a dog of 7 kg. body weight 3 g. of extract reduced the blood pressure reading from 155 to 65 mm. Hg. The reading returned to 100 mm. in 10 minutes then to 138 mm. in 70 minutes and to 142 mm. in 100 minutes.

The fall of blood pressure produced by the drug was not affected by atropine, indicating that the fall was not due to stimulation of the cholinergic nerve endings. After each injection of the drug there was acceleration of the heart beats and of the respiration, probably as a result of the fall in blood pressure (Fig. 5).

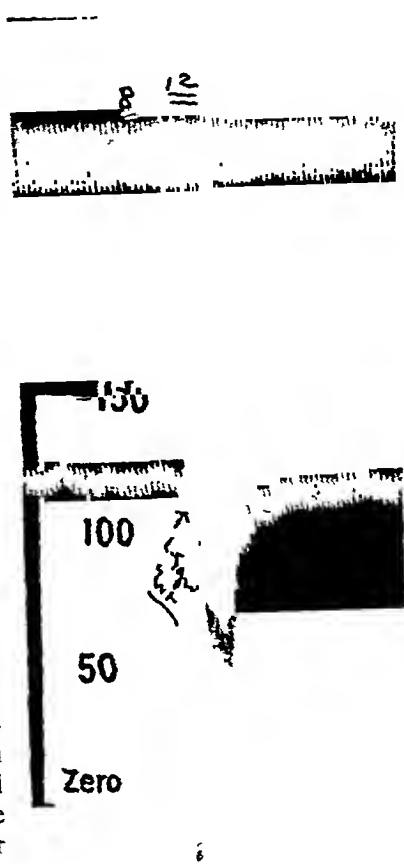


FIG. 5. Dog 7.5 kg. Effect of 1.5 g of extract on respiration (upper tracing) and blood pressure (lower tracing).

Toxicity.—Groups of experimental animals i.e. toads, rats and dogs were injected with relatively large doses of the extract without any toxic effects. The following doses were injected without toxic manifestation. (1) Up to 1.2 g. of extract per 100 g. in the ventral lymph sac of toad. (2) Up to 2 g. of extract intramuscularly per kg. of rat. (3) Up to 0.6 g. of extract intravenously per kg. of dog.

Moreover, intramuscular injections of the extract in rabbits in doses of 0.25 g./kg. every day for 15 days produced no apparent toxic manifestations and no pathological changes in the internal organs on killing the animals.

THE ACTIVE PRINCIPLE

An attempt to isolate the active principle by purifying the extract with lead acetate, and precipitating it from alcoholic solution by ether resulted in an amorphous hygroscopic light brownish powder. This powder was proved to be a glycoside, readily soluble in water or dilute alcohol but insoluble in ether, chloroform, ethyl acetate and absolute alcohol. It had the pharmacological activities of the extract and it formed about 1.6 per cent. of the powdered plant. It did not lose its effects after treatment with hydrochloric acid.

DISCUSSION

Erythraea spicata produces some depression of the force of contraction of the heart in dilutions of 1 in 600 to 1 in 100 of extract. This was not due to vagus stimulation as it occurred after administration of atropine. Dilutions weaker than 1 in 600 of the extract produce practically no depression. In rabbits it produces a definite increase in the coronary flow. It produces definite relaxation of the plain muscle in general. This was shown in: (a) The dilatation of the blood vessels of the toad when perfused with solutions of the drug. The percentage increase in the flow varies from an average of 86 per cent. which was produced by 1 in 100 of the extract, to 19 per cent. which was produced by 1 in 5000 concentration. A moderately good increase in the flow of average 58 per cent. was produced by 1 in 1000. This last concentration did not depress the perfused heart. (b) The powerful relaxation of the rabbits' intestine, in dilutions of 1 in 1000, diminishing both tone and contractions. (c) Definite relaxation of the rabbits' and guinea-pigs' uteri whether pregnant or not.

The drug produces a definite fall of blood pressure; 0.2 g. of the extract per kg. of body weight given intravenously produces an average fall of blood pressure of 46 per cent. of the normal, and 0.04 g. of the extract per kg. of body weight produced an average fall of blood pressure 19 per cent. of the normal. This last dose will make a concentration in the blood of nearly 1 in 2000. This concentration did not depress the perfused heart, so the fall in blood pressure is presumably due to the effect on the blood vessels. The drug is not toxic even if given in relatively large doses. Hydrolysis did not destroy the activity of the drug.

The experiments recorded show that this drug may be of value for reducing blood pressure. At the same time it dilates the coronaries and has a low toxicity, while the effect on the heart in the usual doses is slight. It seems to be worthy of clinical trial as an anti-spasmodic and in cases of hypertension, possibly also in angina pectoris.

SUMMARY

1. The drug produced depression of the toad's heart at a concentration of 1 in 600 of the extract; below this concentration there was practically no depression.
2. It produced increase in coronary flow in the rabbit's heart.
3. It produced fall of blood pressure in dogs and dilatation of vessels in toads.
4. It depressed plain muscle.
5. The active principle is glycosidal in nature.
6. It has a very low toxicity.

Our thanks are due to Professor I. Shawki Bey for suggesting the problem, and to Dr. M. K. Madkour for helping in some of the experiments.

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THE DETERMINATION OF AMINO-COMPOUNDS OCCURRING AS IMPURITIES IN PHARMACEUTICAL CHEMICALS

PART II.

3-AMINO-4-HYDROXYPHENYLARSONIC ACID IN ACETARSOL

By C. W. BALLARD

From the Analytical Control Division of May and Baker, Ltd.

Received November 30, 1948

IN connection with the manufacture of acetarsol, a quantitative method was required for the determination of traces of 3-amino-4-hydroxyphenylarsonic acid and it was thought that a suitable method might be based on the official limit test for this impurity. This depends upon diazotisation and coupling as does the test for arsanilic acid in tryparsamide and hence, in view of the sources of error revealed in the latter¹ and in the quantitative method² proposed by MacDonald and Reynolds, it was decided to make a thorough study of the conditions and reactions involved.

THE OFFICIAL TEST COLOUR STANDARD

The colour standard of the official test is not obtained directly from 3-amino-4-hydroxyphenylarsonic acid but from the product of hydrolysis of acetarsol with hydrochloric acid. Bonino³, who describes a colour test for acetarsol involving heating with hydrochloric acid, suggests that an arsenoso-compound is first formed, whilst Phillips⁴ found that acetarsol yielded 4-bromo-2-aminophenol when heated with hydrobromic

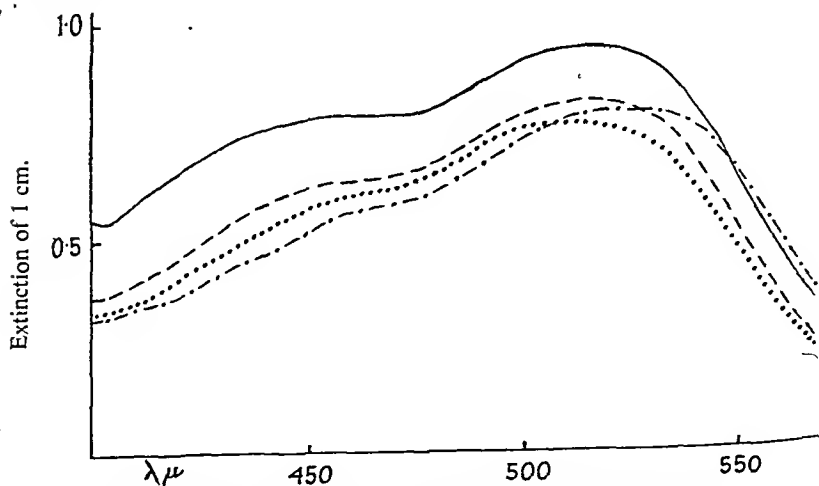


FIG. 1. Spectrophotometric absorption curves of β -naphthol azo-dyes
— 3-amino-4-hydroxyphenylarsonic acid (0.4 mg. = 1.7 micromol)
--- hydrolysis product of acetarsol (0.4 mg. = 1.45 micromol)
..... 2-aminophenol (1.7 micromol)
- . - . 4-chloro-2-aminophenol (1.7 micromol)

DETERMINATION OF AMINO-COMPOUNDS

acid. Hence it appeared desirable to confirm that hydrolysis of acetarsol under the conditions specified in the official test does yield 3-amino-4-hydroxyphenylarsonic acid.

If the arsonic acid group were removed arsenate would be present, but none was detected by a test, using magnesia mixture, of known high sensitivity; more conclusive evidence was obtained from the spectrophotometric absorption curves of the azo-dyes obtained by diazotising and coupling with β -naphthol. From Figure 1 it is seen that the curve for the product of hydrolysis of acetarsol closely follows that for 3-amino-4-hydroxyphenylarsonic acid and the ratio of the colour intensities is approximately that of the molecular weights (mol. wt. of acetarsol/ mol. wt. of 3-amino-4-hydroxyphenylarsonic acid = 1/0.85). On the other hand the curves for 2-aminophenol and 4-chloro-2-aminophenol are somewhat different in shape and moreover the colour intensities are appreciably less than those for an equivalent weight of 3-amino-4-hydroxyphenylarsonic acid. Hence it was concluded that the latter is in fact the product of hydrolysis of acetarsol; however, since it was readily available and a simple method of purifying it by recrystallisation is described by Ehrlich and Berthelm¹, it was used in subsequent work because more convenient.

OPTIMUM CONDITIONS FOR DIAZOTISATION AND COUPLING OF 3-AMINO-4-HYDROXYPHENYLARSONIC ACID

Optimum conditions for diazotisation and coupling were determined in the usual way, an interesting feature of the results being the effect of pH on the rate of coupling and on the colour obtained. With decrease in alkalinity coupling became slower and the violet component of the colour increased gradually but at the point at which β -naphthol began to precipitate a sharp change to a definite violet occurred. These colour changes may be associated with the presence of a hydroxyl group in the dye molecule.

The following method embodies the optimum conditions, and a calibration curve obtained by this method is shown in Figure 3. To 5 ml. of solution containing not more than 0.25 mg. of 3-amino-4-hydroxyphenylarsonic acid add 5 ml. of N 1 hydrochloric acid, cool to about 5°C., add 2 ml. of freshly prepared 0.1 per cent. solution of sodium nitrite and mix. After 2 minutes add 0.05 g. of sulphamic acid, shake well and leave in the ice-bath 5 minutes. Add 10 ml. of previously cooled β -naphthol solution (freshly prepared 5 per cent. solution of recrystallised β -naphthol in 2N sodium hydroxide) and mix. Leave in the ice-bath for 10 minutes, and then place in water at 20°C. for 5 minutes. Read the extinction of 2 cm. using an Ilford 604 filter. Subtract the value of the blank reading obtained in a test omitting the 3-amino-4-hydroxyphenylarsonic acid.

THE OFFICIAL TEST—COPRECIPITATION

Since in the case of trypanamide low results were obtained by pre-cipitating the trypanamide and applying the test for arsenic acid to

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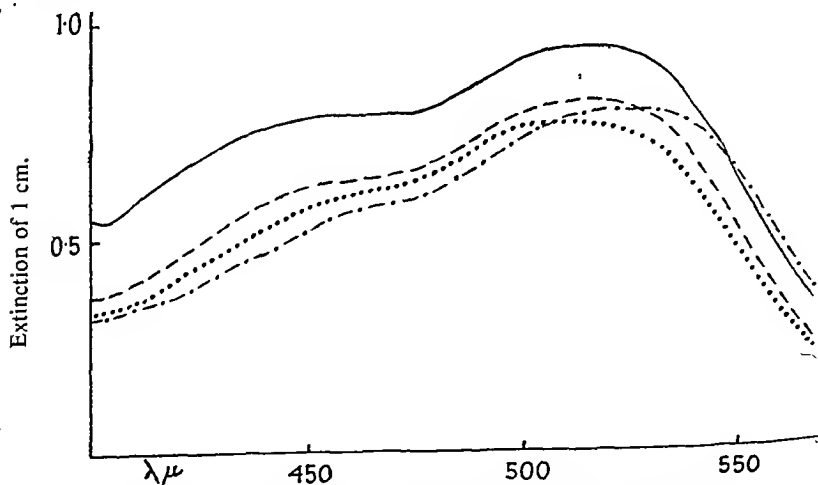


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DETERMINATION OF AMINO-COMPOUNDS

carbonate solution by adding an exactly equivalent amount of diluted hydrochloric acid, followed by 4 precipitations from ice-cold sodium bicarbonate solution with ice-cold diluted hydrochloric acid. The last 2 specimens were tested by the method described above but using varying conditions for effecting solution and precipitation; identical results were obtained on both specimens and are shown in Table I.

TABLE I

Conditions used to dissolve and precipitate acetarsol		Extinction Values
Solution	Precipitation	
a 0.1 g. of sodium bicarbonate in 5 ml. of water at 5°C	5 ml. of N/1 hydrochloric acid at 5°C	0.025
b As above but at room temperature	As above but at room temperature	0.04
c 1 ml. of N/1 sodium carbonate and 4 ml. of water at room temperature	As above	0.055
d 1 ml. of N/1 sodium hydroxide and 4 ml. of water at room temperature	As above	0.07

It was concluded that these two specimens of acetarsol were as pure as could be obtained by the reprecipitation method. Furthermore, it was clear that hydrolysis of acetarsol to 3-amino-4-hydroxyphenylarsonic acid occurs even under relatively mild alkaline conditions and probably also in acid solution. That hydrolysis occurs also in boiling water was shown by recrystallising the purified material from boiling water when the excellent crystals obtained were found to contain about twice as much amino-compound as the original material.

It was now possible to explain the constant value obtained in Figure 2. The extinction value of 0.075 would be made up of about 0.05 from amino-compound produced by hydrolysis during the test itself (Table I, c), and about 0.025 from amino-compound already present in the acetarsol, having been derived from acetarsol by hydrolysis under the conditions of solution and precipitation, and coprecipitated with it.

Calibration curves for 3-amino-4-hydroxyphenylarsonic acid (a) alone, prepared by the method already described, and (b) in the presence of acetarsol, prepared by the proposed method described later, are shown in Figure 3 and it is seen, as expected, that low recovery is obtained in the presence of acetarsol owing to coprecipitation. It is, therefore, necessary to use either internal standards or a calibration curve prepared with acetarsol; for the latter it is sufficient to use a specimen of only relatively low amino-compound content.

PROPOSED METHODS

(a) *Photoelectric absorptiometer.* Prepare a calibration curve by the method described below using, instead of 5 ml. of water, 5 ml. of solution containing up to 0.25 mg. of 3-amino-4-hydroxyphenylarsonic acid; from each value subtract that obtained on the acetarsol alone. The determination on a sample is as follows. To 5 ml. of water at about 5°C. add 0.1 g. of sodium bicarbonate and 0.2 g. of acetarsol. Shake to dissolve and add 5 ml. of N/1 hydrochloric acid previously cooled to about 5°C. Add

the filtrate, it was expected that a similar effect would result with acetarsol. To determine the extent of the loss by coprecipitation in the official test, it was modified slightly as follows to adapt it for photoelectric absorptiometry. Furthermore, sodium carbonate was substituted for sodium hydroxide to avoid hydrolysis of the acetarsol since, in the case of chloracetylaminophenylarsonic acid, appreciable hydrolysis to arsanilic acid had been found to occur in sodium hydroxide solution. Dissolve 0.2 g. in a mixture of 1 ml. of N/1 sodium carbonate and 8 ml. of water. Add 1 ml. of 5N hydrochloric acid, mix, filter, using reduced pressure, to dryness, wash the precipitate with 1 ml. of water and use 1 ml. of water to wash the filter. Cool the mixed filtrate and washings, diazotise and couple with β -naphthol and read the extinction as already described.

This test was applied to a sample of acetarsol which contained about 0.06 per cent. of 3-amino-4-hydroxyphenylarsonic acid; the acetarsol filtered off was then again submitted to the test and this procedure repeated several times. The extinction values (corrected for reagent blank) obtained are shown in Figure 2 and it is seen that the extinction values tend to a constant value of about 0.075, which is shown later to be not entirely due to coprecipitation.

Consideration was then given to devising a test avoiding filtration, especially as it seemed unlikely that the acetarsol would enter into any chemical reactions during the test. Using the same material as used above, optimum conditions were determined and the following provisional test decided upon. Dissolve 0.2 g. in a mixture of 1 ml. of N/1 sodium carbonate and 8 ml. of water and add 1 ml. of 5N hydrochloric acid. Cool to about 5°C., add 2 ml. of freshly prepared 0.5 per cent. solution of sodium nitrate and mix. Complete the test as already described.

Using this method an extinction of 0.44 was obtained, compared with 0.405 for the first value in the precipitation tests (Fig. 2). However, to put the test on a firm basis it was desirable to prepare a sample of pure acetarsol; in addition the constant extinction value of 0.075 reached in the precipitation series required explanation.

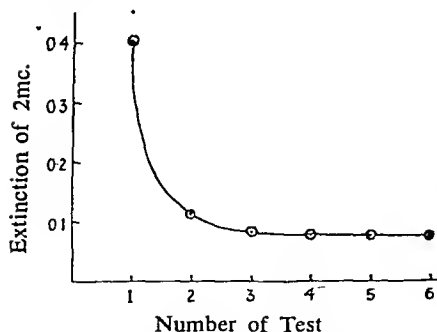


FIG. 2. Results obtained in successive tests by precipitation and filtration on the same material

PURIFICATION OF ACETARSOL AND RECOVERY OF ADDED 3-AMINO-4-HYDROXYPHENYLARSONIC ACID

From Figure 2 it is seen that precipitation by acid from an alkaline solution is a relatively effective method of purifying acetarsol. Material from the same sample as used above was repeatedly submitted to this treatment, 7 precipitations being made at room temperature from sodium

DETERMINATION OF AMINO-COMPOUNDS

3-amino-4-hydroxyphenylarsonic acid by the method described under (a) above (using a 1 cm. cell 0.25 mg. gives about 5 red units). In testing samples, proceed as under (a) but subtract 0.1 red unit from the 1 cm. cell reading before reading off from the calibration curve.

(c) *British Pharmacopœia Limit Test.* The calculated official limit is 0.085 per cent. but, owing largely to the coprecipitation occurring, the actual limit is about 0.13 per cent. Thus the sample used in much of the above work contained by the proposed method 0.06 per cent., whereas by a method closely following the official test as regards preparation of solution for testing only about 0.04 per cent. was found. From Figure 3 it can be calculated that a sample of acetarsol containing 0.13 per cent. of 3-amino-4-hydroxyphenylarsonic acid would give, by the proposed test, a colour equal to that produced by 0.21 mg. of 3-amino-4-hydroxyphenylarsonic acid and hence a simple revised limit test with the same effective limit may be applied as follows. To 5 ml. of water at about 5°C. add 0.1 g. of sodium bicarbonate and 0.2 g. of acetarsol. Shake to dissolve and add 5 ml. of N/1 hydrochloric acid previously cooled to about 5°C. Add 2 ml. of freshly prepared 0.5 per cent. solution of sodium nitrite and mix. After 3 minutes add 0.05 g. of sulphamic acid, shake well and leave in the ice-bath 5 minutes. Add 10 ml. of previously cooled β -naphthol solution and mix. Leave in the ice-bath for 10 minutes and then place in water at 20°C. for 5 minutes. The red colour developed is not greater than that produced in the following way. To 5 ml. of a solution containing 0.21 mg. of 3-amino-4-hydroxyphenylarsonic acid add 5 ml. of N/1 hydrochloric acid and cool to about 5°C. Add 2 ml. of freshly prepared 0.1 per cent. sodium nitrite solution and complete the test as described above.

A solution of 3-amino-4-hydroxyphenylarsonic acid in N/2 hydrochloric acid containing 0.21 mg. in 10 ml. may be prepared by dissolving 0.0124 g. of acetarsol in a mixture of 21 ml. of hydrochloric acid and 21 ml. of water and boiling under a reflux condenser for 5 minutes followed by cooling and dilution with water to 500 ml.

SUMMARY

1. A method has been evolved for the determination of small amounts of 3-amino-4-hydroxyphenylarsonic acid in acetarsol.
2. The official limit test has been examined by photoelectric methods and the actual limit determined. A more satisfactory test is proposed.

My thanks are due to Mr. Bell for the spectrophotometric absorption curves, to Dr. Hersant for helpful criticisms and to the Directors of May and Baker Limited for permission to publish this paper.

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4. Phillips. *J. chem. Soc.*, 1930, 2400.
5. Ehrlich and Bertheim. *Ber. dtsh. chem. Ges.*, 1912, 45, 757.

2 ml. of freshly prepared 0.5 per cent. solution of sodium nitrite and mix. After 3 minutes add 0.05 g. of sulphamic acid, shake well and leave in the ice-bath for 5 minutes. Add 10 ml. of previously cooled β -naphthol solution (freshly prepared 5 per cent. solution of recrystallised β -naphthol in 2N sodium hydroxide) and mix. Leave in the ice-bath for 10 minutes and then place in water at 20°C. for 5 minutes. Read the extinction of 2 cm. using Ilford 604 filter and subtract the value of a reagent blank together with 0.025. Read the amount of 3-amino-4-hydroxyphenylarsonic acid from the calibration curve.

(b) *Tintometer*. Prepare a calibration curve relating red units and

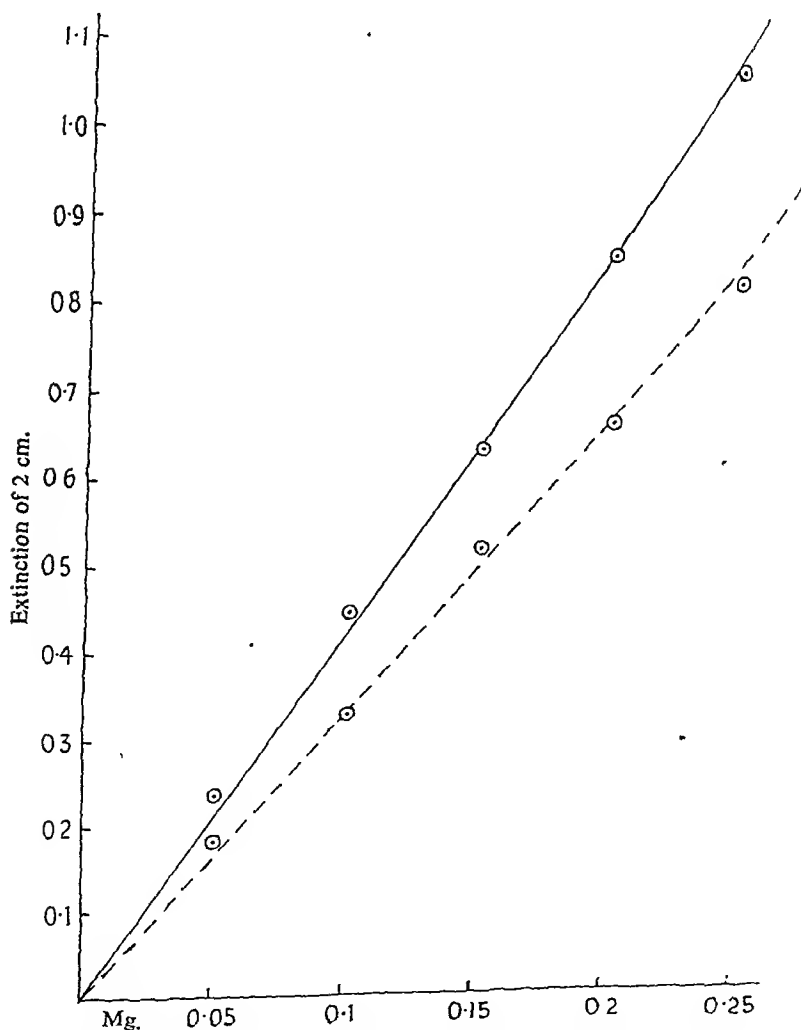
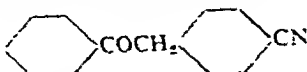
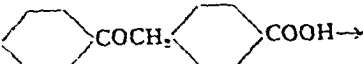
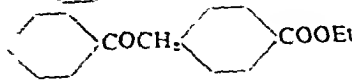
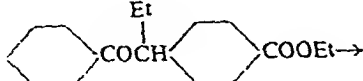
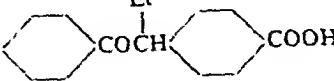


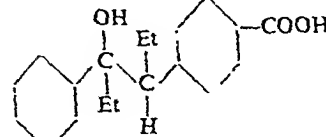
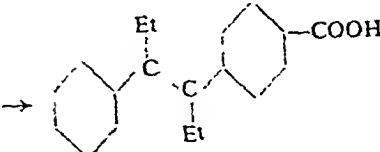
FIG. 3. Calibration curves for 3-amino-4-hydroxyphenylarsonic acid alone

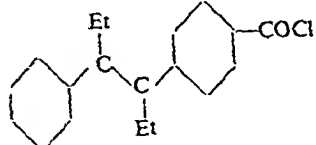
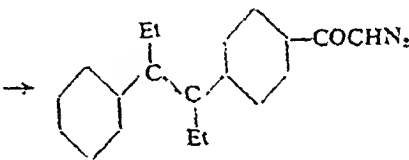
CORTICOSTERONE-LIKE COMPOUNDS

Benzyl cyanide \rightarrow *p*-nitrobenzyl cyanide \rightarrow *p*-nitrophenylacetic acid
 \rightarrow *p*-aminophenylacetic acid \rightarrow *p*-cyanophenylacetic acid \rightarrow *p*-cyano-
 phenacetyl chloride \rightarrow 

\rightarrow  \rightarrow 

\rightarrow  \rightarrow 

\rightarrow  \rightarrow 

 \rightarrow 

COMPOUND II

β -Naphthaleneacetyl carbinol was prepared by a similar method from β -naphthaleneacetic acid and was obtained as a low-melting solid, soluble in boiling water.

Preliminary pharmacological tests, for which we are indebted to Professor Buttle and Dr. Dyer of the Pharmacological Department of this School, indicate that 4-(ω -hydroxyaceto) α : β -diethylstilbene (II) is less active than its corresponding 4'-hydroxy derivative. The naphthaleneacetyl carbinol was found to be inactive at the dose levels used.

This work is being extended to cover other derivatives of naphthalene and hydroxyaceto derivatives of such other systems as may be in any way considered as fragments of the cyclopentenophenanthrene nucleus, including a thorough exploration of the indene skeleton from this point of view.

EXPERIMENTAL

4-Cyanodesoxybenzoic acid.— $C_6H_5.CO.CH_2.C_6H_4.CN$

p-Nitrobenzyl cyanide was obtained in 57 per cent. yield, converted into the corresponding acid (yield 90 per cent.) which was then reduced to *p*-aminophenylacetic acid (yield 85 per cent.) according to the method of Robertson^{4,5,6}. This compound was converted into *p*-cyanophenylacetic acid by following the process of Jaeger and Robinson⁷ when a 59 per cent. yield was realised. 5 g. of phosphorus trichloride was added to 10 g. of dry *p*-cyanophenylacetic acid and the mixture heated on a water-bath till completely liquid. While still warm, 50 ml. of dry benzene

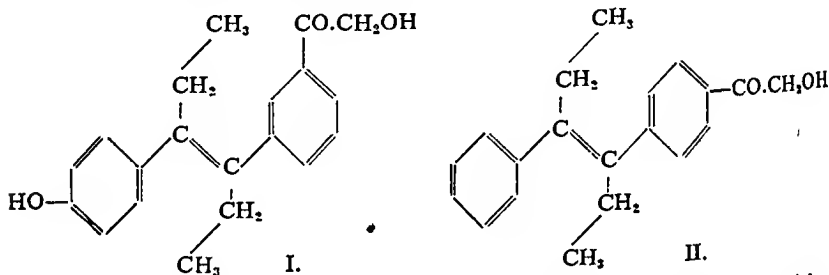
SYNTHETIC COMPOUNDS POSSESSING CORTICOSTERONE-LIKE ACTIVITY

BY R. A. KHAN AND W. H. LINNELL

*From the Pharmaceutical Chemistry Research Laboratories, the School of
Pharmacy, University of London*

Received November 16, 1948

It has been shown by Linnell and Roushdi¹ that 4-hydroxy-3'-(ω -hydroxyaceto) α : β -diethylstilbene (I) possesses approximately 1/200th the activity of desoxycorticosterone, and also that benzoyl carbinol C_6H_5 .COCH₂.OH exhibits small cortical activity whilst *p*-hydroxybenzoyl carbinol, $OH.C_6H_4.CO.CH.OH$ is completely devoid of activity. It was, therefore, decided to prepare 4-(ω -hydroxyaceto) α : β -diethylstilbene (II) and see if the absence of the hydroxy group in the stilbene compound would enhance activity.



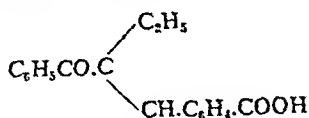
The keto-alcohol group in the new compound is in the 4-position instead of the 3- and this may also have the effect of increasing activity, as in the synthetic hormones derived from the diethylstilbene molecule the 4-position seems to afford the more active compounds.

The compound in question 4-(ω -hydroxyaceto) α : β -diethylstilbene was prepared according to the scheme on page 231.

4-Cyanodesoxybenzoin was obtained by treating *p*-cyanophenacetyl chloride with benzene in the presence of aluminium chloride according to the method of Allen and Barker² for preparing desoxybenzoin. The product was hydrolysed, by boiling with sulphuric acid, to 4'-carboxydesoxybenzoin, the ethylation of which at the α -position was achieved after a preliminary esterification and the α -ethyl ester was then saponified by alcoholic caustic potash to give 4-carboxy- α -ethyl-desoxybenzoin. By treatment with ethyl magnesium iodide, 3-phenyl-4-(*p*-carboxyphenyl)hexane-3-ol was obtained which was not isolated in a pure state. This compound was dehydrated by heating with iodine when the 4-carboxy- α : β -diethylstilbene obtained was converted into 4-(ω -hydroxyaceto) α : β -diethylstilbene by the method of Steiger and Reichstein³ in which the appropriate acid chloride was treated with diazomethane and the diazoketone so produced decomposed with 2N sulphuric acid. 4-(ω -hydroxyaceto) α : β -diethylstilbene was obtained in 40 per cent. yield as a bright yellow viscid oil boiling at 175° to 180°C. at 5 mm. Hg.

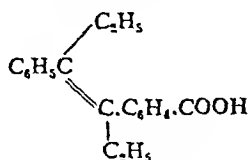
CORTICOSTERONE-LIKE COMPOUNDS

4'-Carboxy- α -ethyl-desoxybenzoin.—



2.1 g. of 4'-carbethoxy- α -ethyl-desoxybenzoin was saponified with 110 ml. of N/10 alcoholic potassium hydroxide solution. The crude oily substance was crystallised from 70 per cent. acetic acid, when fine white crystals of 4'-carboxy- α -ethyl-desoxybenzoin melting at 126° to 127°C. were obtained. Yield 1.42 g. (75 per cent.). Found: C, 75.98; H, 5.95 per cent.: $\text{C}_{17}\text{H}_{16}\text{O}_3$ requires C, 76.11; H, 5.97 per cent.

4'-Carboxy- α : β -diethylstilbene.—



A cold ethereal solution of ethyl magnesium iodide (prepared by adding 1.65 g. of ethyl iodide to 0.25 g. of dry magnesium in ether) was added slowly with frequent shaking to a solution of 2.7 g. of 4'-carboxy- α -ethyl-desoxybenzoin in dry ether. The reaction mixture, protected from moisture, was left overnight and then refluxed for 3 hours. The resulting product was decomposed with ice and dilute sulphuric acid, and the product worked up in the usual way after which the oily residue was heated on a water-bath with 1 to 2 per cent. of iodine under an air condenser for 1 hour. The residue, dissolved in ether, was washed with water and 5 per cent. sodium thiosulphate solution and dried overnight. The solvent was removed by distillation and 2 g. of a dark brown oil was obtained. From boiling 90 per cent. acetic acid, on scratching the sides of the vessel and standing, it gave a white powder melting at 95° to 100°C. Titration with N/125 sodium hydroxide indicated a purity of 96.1 per cent. Its acid chloride prepared as described below gave a crystalline 4-amido-derivative melting at 94°C. Found: N, 4.79 per cent.; $\text{C}_{19}\text{H}_{11}\text{ON}$ requires N, 5.01 per cent.

4-(ω -Hydroxyaceto)- α : β -diethylstilbene.— $\text{C}_{18}\text{H}_{19}\cdot\text{COCH}_2\text{OH}$

1.5 g. of 4-carboxy- α : β -diethylstilbene was converted into its acid chloride by boiling with 5 ml. of dry benzene and 5 ml. of thionyl chloride. The acid chloride in dry ether was treated with an ethereal solution of diazomethane (prepared from 10 g. of nitrosomethylurea) and the mixture kept at 6°C. for 1 hour and then allowed to stand overnight at room temperature. After removing the excess of diazomethane and the solvent, the diazoketone, obtained as a dark oil, was dissolved in 10 ml. of dioxan and, on addition of 15 ml. of 2N sulphuric acid, nitrogen was liberated during about half an hour, after which the reaction mixture was warmed to 40° to 45°C. when no more gas was evolved. It was then diluted with water and extracted with ether. The ethereal solution was washed

was added and the benzene solution decanted on to 10 g. of anhydrous aluminium chloride. When the spontaneous reaction had subsided the mixture was refluxed for 1 hour, cooled and poured into a solution of 25 ml. of concentrated hydrochloric acid in 500 ml. of cold water. The benzene layer was separated, the aqueous portion was washed with benzene and the washings combined with the benzene solution. The solvent was removed by distillation and the yellow sticky residue was crystallised from a small quantity of alcohol (97 per cent.) when 4'-cyanodesoxybenzoin was obtained as light yellow crystals melting at 105° to 106°C. Yield 5.7 g. (41 per cent.). Found: C, 80.1; H, 5.0; N, 6.33 per cent.; $C_{15}H_{11}OH$ requires C, 81.4; H, 4.9; N, 6.35 per cent.

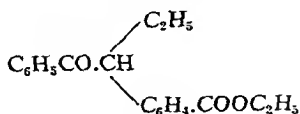
4'-Carboxydesoxybenzoin.— $C_6H_5.CO.CH_2.C_6H_4.COCH$

1 g. of 4'-cyanodesoxybenzoin was refluxed with 25 ml. of glacial acetic acid, 25 ml. of water and 25 ml. of concentrated sulphuric acid for 5 hours and poured into an equal volume of cold water. The thick white precipitate was collected, washed with cold water and crystallised from 60 per cent. acetic acid. After purification by animal charcoal and recrystallisation from 60 per cent. acetic acid, 4'-carboxy-desoxybenzoin was obtained as shining white flakes melting at 209°C. (decomp.). Yield 0.75 g. (63 per cent.). Found: C, 74.3; H, 5.1; per cent. $C_{15}H_{12}O_3$ requires C, 75.0; H, 5.0 per cent.

4'-Carbethoxydesoxybenzoin.— $C_6H_5.CO.CH_2.C_6H_7.COOC_2H_5$

4'-Carboxydesoxybenzoin was esterified by bubbling hydrogen chloride through an alcoholic solution of 3 g. of the acid and refluxing the mixture at the same time when a quantitative yield of 4'-carbethoxydesoxybenzoin was obtained as pale shining flakes melting at 106°C. Found C, 75.1; H, 6.02 per cent.; $C_{17}H_{16}O_3$ requires C, 76.1; H, 5.97 per cent.

4'-Carbethoxy- α -ethyl-desoxybenzoin.—



2.5 g. of 4'-carbethoxydesoxybenzoin was dissolved in 10 ml. of boiling absolute alcohol and to the boiling solution 0.5 g. of sodium in 5 ml. of absolute alcohol and 3.3 g. of ethyl iodide were added; the mixture being heated until neutral to litmus. A further quantity of 0.5 g. of sodium in 5 ml. of absolute alcohol and 3.3 g. of ethyl iodide was added and the mixture again refluxed until neutral. After cooling and dilution with water, the alcohol was removed under reduced pressure. The oily suspension was extracted with ether and the ethereal solution well washed with water, 5 per cent. sodium thiosulphate solution and water. After drying overnight the ether was removed by distillation and 4'-carbethoxy-ethyl-desoxybenzoin was obtained as a bright yellow oil which solidified to a glassy substance. Yield 2.1 g. (90 per cent.). Found: C, 77.0; H, 6.79 per cent.; $C_{19}H_{20}O_3$ requires C, 77.02; H, 6.75 per cent.

SYNTHESIS OF MONOMERIC FORMS OF α -HYDROXY- β -METHOXYPROPIONALDEHYDE AND 1:3-DIMETHOXY-PROPANONE-2

BY YEHYA M. ABOUZEID AND W. H. LINNELL

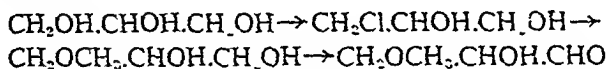
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THE compounds concerned in this communication are required for the synthesis of methylated hexoses which could not possess a pyranose structure.

α -Hydroxy- β -methoxypropionaldehyde had not been obtained in the monomeric form, although the dimeride was prepared by Fisher and Bear¹ and proved to be a crystalline substance melting at 120 to 121°C. which on heating with pyridine suffered rearrangement into the monomethyl ether of dihydroxyacetone. Alkaline condensation of this dimeride gave rise to sugars with branched chains.

The monomeric form was prepared by the following series of reactions:



The α -monomethylether of glycerol was obtained by the gradual addition of a methyl alcoholic solution of glycerol- α -monochlorohydrin to a solution of sodium methylate, the mixture being refluxed until separation of sodium chloride ceased: it was obtained as a syrup boiling at 135° to 136°C./40 mm. Hg. pressure. The oxidation was attempted *via* the Oppenauer² reaction using aluminium tertiary butoxide as this method was successful in the oxidation of cholesterol to cholestenone as applied by Adkins and Cox³. The conditions of Adkins and Cox were used, but a very poor yield was obtained.

Fenton and Jackson⁴ originated the hydrogen peroxide-ferrous sulphate oxidation of polyhydric alcohols. This reagent appears to be specific for the primary alcoholic groups, no instance of ketone formation being reported. It has been successfully used for the oxidation of ethylene glycol to glycol aldehydes, mannitol to mannose⁵ and glycerol to glyceraldehyde. After numerous trials satisfactory conditions were established for the oxidation of the α -monomethylether of glycerol, and the product on isolation gave a positive test with Schiff's reagent, reduced Fehling's and ammoniacal silver nitrate solutions, gave an immediate precipitate with 2:4-dinitrophenylhydrazine and a solid bisulphite compound. The product obtained with 2:4-dinitrophenylhydrazine, melted at 234° to 236°C. (with decomposition) and gave analytical figures in agreement with those of the osazone of α -hydroxy- β -methoxypropionaldehyde. The formation of the osazone proves the

with water and a small quantity of 1 per cent. sodium bicarbonate solution. After drying the ethereal solution, ether was removed by distillation and the dark oil distilled under vacuum. 4-(ω -Hydroxyaceto)- α : β -diethylstilbene was obtained as a yellow oil boiling at 175° to 180°C. / 5 mm.Hg. Yield 0.8 g. (40 per cent.). Found C, 78.8; H, 7.5 per cent. : $C_{20}H_{22}O_2$ requires C, 81.2; H, 7.6 per cent. It was soluble in ether, alcohol, acetone, benzene, chloroform and toluene, but insoluble in water. It reduced cold ammoniacal silver nitrate solution and gave an osazone with 2:4-dinitrophenylhydrazine, which, on recrystallisation from toluene, melted at 115°C. Found: N, 17.5 per cent.; $C_{32}H_{28}O_8N_8$ requires N, 17.18 per cent.

β -Naphthaleneacetyl carbinol.— $C_{10}H_7 \cdot CH_2 \cdot CO \cdot CH_2 \cdot OH$

An ethereal solution of β -naphthaleneacetyl chloride obtained from 1 g. of β -naphthaleneacetic acid was treated with diazomethane as described earlier. The resulting diazoketone was dissolved in 10 ml. of dioxan, decomposed with 5 ml. of 2N sulphuric and worked up as stated previously to give 0.71 g. (63 per cent.) of β -naphthaleneacetyl carbinol melting at 90° to 93°C. as light yellow crystals from 97 per cent. alcohol. Found: C, 75.32; H, 5.91 per cent.; $C_{13}H_{12}O_2$ requires C, 78.00; H, 6.00 per cent.

It was soluble in most organic solvents and also in boiling water. It reduced ammoniacal silver nitrate solution in the cold. With 2:4-dinitrophenylhydrazine it gave the corresponding hydrazone melting at 24°C. The hydrazone on analysis gave N, 14.0 per cent.; $C_{19}H_{16}O_8$ requires N, 14.7 per cent.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Alkaloids of the Australian Rutaceae. G. K. Hughes, F. N. Lahey, J. R. Price and L. J. Webb. (*Nature*, 1948, 162, 223.) As part of a survey of the occurrence and pharmacology of alkaloids in the Australian flora, three Queensland rain-forest species have been examined. The bark of *Melicope farcana* F. Muell. (bark) contained melicopine ($C_{17}H_{15}O_5N$), pale yellow needles, m.pt. 178.5° to $179.5^\circ C$. melicopidine ($C_{17}H_{15}O_5N$), pale yellow prisms, m.pt. 121° to $122^\circ C$. melicopicine, yellow prisms, m.pt. 131° to $132^\circ C$. acronycidine ($C_{15}H_{15}O_5N$), colourless needles, m.pt. 136.5° to $137.5^\circ C$. While the leaves of *M. farcana* also contained melicopine, melicopidine and melicopicine, acronycidine was absent. Another colourless alkaloid was present but has not yet been obtained pure. The bark of *Acronychia baueri* Schott contained acronycine ($C_{20}H_{17}O_5N$), pale yellow needles, m.pt. 174° to $175^\circ C$. melicopine, melicopidine and acronycidine, while melicopicine was also isolated from the leaves. The bark of *Evodia xanthoxyloides* F. Muell. contained evoxanthine ($C_{16}H_{13}O_4N$), pale yellow needles, m.pt. 217° to $218^\circ C$. and melicopidine. It has been established that the five yellow alkaloids are N-methyl acridones. Acronycidine (colourless) is considered to be a quinoline derivative related to skimmianine.

F. H.

ANALYTICAL

Arsenic and Iron, Volumetric Microdetermination of. G. F. Smith and J. S. Fritz. (*Anal. Chem.*, 1948, 20, 874.) The equivalence point for the potentiometric titration of 0.001N solutions of ferrous iron, oxalic and arsenite ions in 2F perchloric acid solutions by oxidation using 0.001N solutions of perchloratoceric acid in the same acid medium covers the range 0.95 to 1.5 volts, and the ratio of change in potential to oxidant addition is greater at 1.23 volts. Hence, by using a redox indicator, ferrous nitrophenanthroline ion (nitroferroin) having a transition potential of 1.23 volts in 2F perchloric acid the more laborious potentiometric titration procedure is avoided. The colour change is red to faint blue. Results are accurate to ± 0.5 per cent. In the case of iron, results obtained by the above method and the spectrophotometric determination are of comparable accuracy.

E. N. I.

Capsaicin, Assay of. J. Büchi and F. Hippenmeier. (*Pharm. Acta Helvet.*, 1948, 23, 327.) In testing capsicum preparations by taste, it is found that the sensitivity of different individuals shows very great variations, the sensitivity of the tongue rapidly decreases, and recovery of the sensitivity requires about 3 hours. A new method of assay is based on a study of the properties of pure capsaicin. Chemically, capsaicin is a vanillylamine acylated with a decylenic acid. The most important groupings are a double bond, an acid amide grouping, a free phenolic hydroxyl, and a methylated phenolic hydroxyl group. Attempts to base a method of assay on the reactions of one of these groupings were unsuccessful for various reasons. A method based on the reducing power of capsaicin is as follows: about 10 mg. of

ethereal extracts being washed with sodium bicarbonate solution, with water, and then dried over anhydrous sodium sulphate. On removal of the ether 10 g. of a colourless syrup remained. After precipitation of chromium salts with sodium bicarbonate a further 15 g. of colourless syrup was obtained from the mother liquors by extraction with absolute alcohol. The combined syrups were then fractionated under reduced pressure when a main fraction boiling at 40° to 42°C./22 mm. Hg. pressure was obtained. On redistillation this fraction yielded 20 g. of 1:3-dimethoxypropanone-2. Yield, 40 per cent. Found: C, 49.76; H, 8.44 per cent.; $C_5H_{10}O_3$ requires C, 50.85; H, 8.47 per cent. The product gave positive qualitative reactions of a ketone.

1:3-Dimethoxypropanone-2-2':4'-dinitrophenylhydrazone.

$(CH_3O.CH_2)_2C:N.NH.C_6H_3(NO_2)_2$.—A solution of 2 g. of 1:3-dimethoxypropanone-2 in 5 ml. of absolute alcohol was mixed with a filtered solution of 2:4-dinitrophenylhydrazine (2 g. in 30 ml. of absolute alcohol plus 1 ml. of concentrated sulphuric acid). The mixture was warmed and, on cooling, yellow needle-shaped crystals separated. After two recrystallisations from boiling alcohol and drying *in vacuo* the melting point was found to be 112° to 113°C. Found: C, 44.66; H, 4.75; N, 18.8 per cent.; $C_{11}H_{14}O_6N_4$ requires C, 44.30; H, 4.70; N, 18.79 per cent.

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The use of infra-red rays is quicker and more satisfactory. The lamp, placed at a distance of 37 cm. from the material, raises its temperature to about 62°C., and drying is generally complete in 1 to 2 hours, when the extract is spread out in a layer of 1 to 2 mm. thickness and 30 sq. cm. area. Even in this case loss of weight continues after all the moisture has been removed. The presence of residual traces of water may be detected by using the moisture-detecting powder recently described (Baymond, *Pharm. Acta Helvet.*, 1948, 23, 207; *J. Pharm. Pharmacol.*, 1949, 1, 44), the extract being placed in a small crucible heated in an oil-bath at 130°C., and covered with a watch glass which has a coating of the water-detecting powder. The dry extracts examined contained in general less than 3 per cent. of moisture. Figures of over 10 per cent., sometimes recorded in the literature, are erroneous.

G. M.

Glycerol, Colorimetric Method for the Determination of Small Quantities of. V. H. Mikkelsen. (*Analyst*, 1948, 73, 447.) The method used depends on the fact that glycerol is oxidised by bromine to 1:3-dihydroxyacetone and probably also to glyceraldehyde; addition of concentrated sulphuric acid yields methyl glyoxal which will react with codeine, thymol, resorcinol and β -naphthol to form coloured compounds. Codeine was chosen for the reaction as it gave a blue colour with a characteristic absorption band with a maximum at 6600Å. By systematic variation of (1) the amount of bromine, (2) the glycerol concentration, and (3) the heating time both for the oxidation and for the codeine reaction, a procedure was obtained which gave reproducible results and proportionality between the extinction coefficient and the glycerol concentration. Details of the final colorimetric method are given, together with details of the method as applied to the determination of glycerol in a morphine injection. In place of saturated bromine water an aqueous solution of potassium bromate and bromide containing an equal volume of 2N hydrochloric acid was used.

R. E. S.

Hyoscyne and Hyoscyamine, Separation of, and the Alkaloidal Assay of *Duboisia* spp. E. M. Frautner and M. Roberts. (*Analyst*, 1948, 73, 140.) Hyoscyne and hyoscyamine, including atropine, occur together in several solanaceous plants, and are generally determined together as total alkaloids, and calculated in terms of hyoscyamine. Two methods of separation of the two alkaloids are described, the first, an approximately quantitative separation of the alkaloids by chromatographic adsorption on activated silica, and subsequent fractional elution, and the second, the separation and identification of the components of a mixture of alkaloids by fractional precipitation of their picrates. Hyoscyne and hyoscyamine are both strongly adsorbed on a silica column from benzene solution, and are separated by elution with absolute alcohol. The hyoscyne is removed rapidly but the hyoscyamine only slowly. By adding a trace of dimethylamino-azo-benzene (butter yellow, dimethyl yellow, C.I.19) to the benzene the alkaloids can readily be detected on the column. The dye is only weakly adsorbed by the silica, giving a brilliant red colour, except where the alkaloids are preferentially adsorbed; here a yellow band of unadsorbed dye is left. The dye is quickly removed by elution with ether or absolute alcohol, but if these solvents are then replaced by benzene or, better, light petroleum, containing a little of the dye, the adsorption zones reappear. It can then be seen, which zones have been eluted, separated or spread. The efficiency of the separation of the alkaloids is dependent on the dimensions of the silica column, the authors used a column 12 cm. x 1 cm. The best results are obtained when the ratio of the amount of hyoscyne to

capsaicin is dissolved, with gentle warming, in 45 ml. of 0.1N sodium hydroxide, and made up to 50 ml. with the alkali; 5 ml. of this solution is treated with 3 ml. of reagent (3 g. of pure phosphomolybdic acid in 100 ml. of water), and, after standing for 1 hour, is examined colorimetrically in a 0.5 cm. cell, using filter S72. A standardisation curve is prepared with pure capsaicin. This method has not yet been extended to the assay of capsicum itself.

G. M.

Capsaicin in Drugs, Determination of. J. B ü c h i and F. H i p p e n - m e i e r. (*Pharm. Acta Helvet.*, 1948, 23, 353.) The method is based on the colorimetric reaction for capsaicin previously described by the authors (*Pharm. Acta Helvet.*, 1948, 23, 327). Details are as follows: 5g. of powdered capsicum is shaken for 30 minutes with 50 ml. of dilute alcohol, and the mixture is filtered into a 250-ml. beaker, the filter being washed with 20 ml. of dilute alcohol. After the addition of 15 ml. of 0.5N sodium hydroxide to the filtrate, the mixture is warmed on the water-bath, with occasional stirring, for about 1 hour until the alcohol is removed and the mixture does not froth on stirring. The solution is cooled and treated with 2N hydrochloric acid, added drop by drop, until it does not turn thymol blue paper blue but still reacts alkaline to litmus paper. This solution, which may be slightly turbid, is then shaken out with 50, 25, and 25 ml. of ether. The combined clear ether extracts are dried with sodium sulphate, and the ether is removed on the water-bath, the residue being dried for 30 minutes in a current of air. The residue is dissolved by gentle heat in 20 ml. of (exactly) 0.1N sodium hydroxide, used in two portions, and the solution is strained and made up to 25 ml. with the alkali. This solution should be clear or at most slightly opalescent. 5-ml. portions are transferred to two 20-ml. measuring flasks, and to a 20-ml. stoppered tube. To each of the flasks, is added 3 ml. of reagent (3 g. of phosphomolybdic acid in water to 100 ml.), while 3 ml. of water is added to the tube. After 1 hour, both flasks are made up to the mark with alcohol (95 per cent.), and shaken vigorously, final adjustment of the volume being made after cooling. The contents of the tube are diluted similarly to 20 ml. The extinction of the liquids in the flasks is then measured against that in the tube, using a 0.5 cm. cell and filter S72. The amount of capsaicin in mg./5ml. of the 0.1N sodium hydroxide solution is then equal to the extinction co-efficient $\times \frac{2.5}{1.100}$. The figures obtained from a number of samples of the official Swiss drug (*Capsicum annuum*) range from 0.122 to 0.304 per cent. One sample of cayenne showed 0.415 per cent. In applying the method to the tincture, 50 g. of the latter is shaken out with 50 ml. of light petroleum the extract being washed with 3 quantities, each of 25 ml. of dilute alcohol. The combined alcoholic solutions are combined with the original tincture which has been extracted with light petroleum and, after the addition of 15 ml. of 0.1 N sodium hydroxide, the method is continued as before. For concentrated fluid extract of capsicum (oleoresin of capsicum), 1 g. of the material is dissolved in 40 ml. of light petroleum and this solution is shaken out 3 times with 20 ml. quantities of dilute alcohol. The combined alcoholic solutions are treated as before.

G. M.

Extracts, Determination of Moisture in, by Infra-red Heating. M. Bouchardy and A. Mirimanoff. (*Pharm. Acta Helvet.*, 1948, 23, 321.) The determination of the moisture content of dry extracts by heating in an oven is unsatisfactory, as constant weight is never obtained.

The use of infra-red rays is quicker and more satisfactory. The lamp, placed at a distance of 37 cm. from the material, raises its temperature to about 62°C., and drying is generally complete in 1 to 2 hours, when the extract is spread out in a layer of 1 to 2 mm. thickness and 30 sq. cm. area. Even in this case loss of weight continues after all the moisture has been removed. The presence of residual traces of water may be detected by using the moisture-detecting powder recently described (Baymond, *Pharm. Acta Helvet.*, 1948, 23, 207; *J. Pharm. Pharmacol.*, 1949, 1, 44), the extract being placed in a small crucible heated in an oil-bath at 130°C., and covered with a watch glass which has a coating of the water-detecting powder. The dry extracts examined contained in general less than 3 per cent. of moisture. Figures of over 10 per cent., sometimes recorded in the literature, are erroneous. G. M.

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that of hyoscyamine lies between the limits 1:4 and 6:1. The fractions obtained are pure enough to give crystalline picrates, even when only 1 or 2 mg. of alkaloid is present. The picrates are prepared by the addition of a dilute solution of picric acid, followed by crystallisation from chloroform, and are readily identified by their m.pt., hyoscine picrate usually melts at the correct temperature, 187° to 188°C., but the hyoscyamine picrate is frequently less pure and may melt several degrees lower than the correct value, 165° to 166°C. The separation of hyoscine and hyoscyamine picrates is too slow to replace the better and more rapid fractionation which can be achieved with a silica column, it is, however, useful if it is necessary to establish the identity of the main alkaloid present in the hyoscyamine fraction. Using these methods to assay a sample of *Duboisia myoporoides* the authors found that the leaves contained about 2 per cent. of hyoscyamine, 0.7 per cent. of hyoscine, and 0.2 per cent. of other alkaloids. Detached leaves of *Atropa belladonna*, starved to the point of proteolysis, assayed by similar methods, showed that the hyoscyamine content remained unchanged, and that neither hydrolysis nor demethylation takes place to any appreciable extent.

L. H. P.

Inositol, Chemical Determination of. P. Fleury and A. Leconles. (*C. R. Acad. Sci., Paris*, 1948, **227**, 691.) The method depends upon measuring the volume of carbon dioxide released when inositol is treated with periodic acid. Though several days are necessary for complete evolution, the majority is released within a short time and this quantity is proportional to the concentration of inositol. Interfering substances are first destroyed by treating with magnesia at 100°C. Experiments with known concentrations of inositol added to urine showed that an accuracy of about ± 2.5 per cent. was normally obtained, with a maximum error of ± 10 per cent.

J. W. F.

Iodine in Organic Compounds, Micro-determination of. R. Grangaud. (*Ann. pharm. Franc.*, 1948, **6**, 212.) The method is based on the reaction of iodide, in solution, with silver iodate, forming soluble iodate. The organic compound is decomposed in a tube in a current of oxygen, the gases evolved being passed over heated platinum foil. The iodine is absorbed in 0.75 ml. of 0.2N sodium hydroxide containing 5 drops of 30 per cent. hydrogen peroxide. After the combustion, the hydrogen peroxide is destroyed by heating for 5 minutes on the water-bath, methyl red is added, and the liquid is adjusted to a pH of about 5. It is then cooled in ice and treated with about 50 mg. of solid silver iodate, and shaken vigorously for 1 minute. The mixture is filtered by syphoning (Pregl method) through a sintered glass micro-filter covered with a layer of asbestos. The filter is washed with 0.5 ml. of dilute alcohol, then twice with alcohol (95 per cent.). The filtrate is diluted with water to about 30 ml. and treated with a few crystals of potassium iodide and 1 ml. of 10N sulphuric acid. After 1 minute, the liberated iodine is titrated with 0.02N iodine, using starch as indicator. Owing to the formation of traces of nitrous acid, the method must be modified for the assay of nitrogenous substances or for liquids which are sealed into a capillary in presence of a crystal of ammonium nitrate. In this case the hydrogen peroxide is omitted, and after the combustion the liquid in the absorber is treated with 5 drops of sodium bisulphite solution and 0.2 ml. of N sulphuric acid. After heating on the water-bath for 10 minutes to destroy the nitrous acid, the liquid is cooled and treated with 5 drops of hydrogen peroxide and 0.2 ml. of N sodium hydroxide. The assay is then continued as before.

G.-M.

Iron, Colorimetric Determination of, with isoNitrosodimethyldihydroresorcinol. S. C. Shome. (*Anal. Chem.*, 1948, 20, 1205.) A spectrophotometric study of the colorimetric determination of iron (ferrous or ferric) has been made using the blue colour given with isonitrosodimethyldihydroresorcinol. The effect of concentration of the reagent was studied together with problems of iron concentration, colour stability, and interference of other ions. The colour formed was found to be stable and both ferrous and ferric iron reacted; iron could thus be determined in the presence of comparatively large amounts of nickel, cobalt, phosphate, arsenate, fluoride, oxalate, citrate, tartrate, borate, and perchlorate in slightly acid medium. Sensitivity measurements indicated that iron can be detected with this reagent to an extent of 1 part in 50,000,000 parts of solution. Analyses of the purified iron-isonitrosodimethyldihydroresorcinol complex showed that an iron atom combines with three molecules of the organic reagent; the complex retained two molecules of water even after drying in a vacuum desiccator over sulphuric acid for a number of days.

R. E. S.

Œstrogens, Synthetic, Colorimetric Determination of. A. Carayon-Gentil and M. J. Cheymol. (*Ann. pharm. Fr.*, 1948, 6, 129.) Methods are given for the determination of stilbœstrol, hexœstrol and dienœstrol in tablets and oily solutions. *Extraction of tablets:* the finely powdered material, corresponding to 1 to 3 mg. of œstrogen, is shaken mechanically with two quantities of ethyl acetate, 40 ml. in all, each shaking being continued for 1 hour. The mixture is filtered, and the residue washed with water. After evaporation of the solvent, the residue is taken up in methyl alcohol. If the tablets are prepared with esters of the œstrogens, a saponification must follow the extraction. *Oily solutions:* 1 ml. of solution, containing 1 to 2 mg. of œstrogen, is refluxed with 15 ml. of 5N methyl alcoholic solution of potassium hydroxide until saponification is complete. After diluting with 4 volumes of water, unsaponifiable matter is removed by shaking twice with light petroleum, and the aqueous phase is acidified and extracted with ether. The ethereal solution is washed with sodium carbonate solution (1 per cent.) then with water. The ether is removed by evaporation, and the residue is taken up in methyl alcohol so that 1 ml. of the solution corresponds to 50 to 100 μ g of œstrogen. For dienœstrol a different method is employed: 1 to 2 ml. of solution, containing 2 to 5 mg. of dienœstrol, is shaken mechanically for 30 minutes with 20 ml. of methyl alcohol, and the extraction is repeated with a further quantity of methyl alcohol. The combined methyl alcoholic solution is filtered and used for the determination. *Determination of dienœstrol.* A coupling method is used. One ml. of the methyl alcoholic solution is mixed with 8 ml. of borate buffer solution, pH 12 (20 g. of boric acid and 280 ml. of N sodium hydroxide per l.) heated for 6 minutes at 80°C., then cooled to 20°C. It is then treated with 2 ml. of diazotised sulphanilic acid solution and, after 5 minutes, the colour is compared with that obtained from a standard solution of dienœstrol. Dienœstrol from oily solutions may give a final solution which is slightly turbid. It can be cleared by adding 1 or 2 drops of a 10 per cent. solution of calcium chloride.

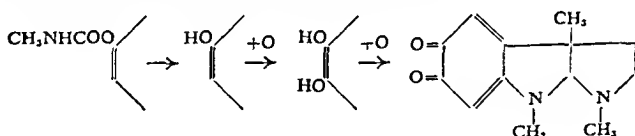
G. M.

Pentaerythrityl Tetranitrate and Glyceryl Trinitrate, Determination of. J. Allert. (*Dansk Tidsskr. Farm.*, 1948, 22, 188.) By saponification of alkyl nitrates with alkali, nitrites are formed and may be determined colorimetrically with e.g. procaine and α -naphthylamine. This method is

however not satisfactory for pentaerythrityl tetranitrate. After *trans*-esterification with sulphuric acid, the nitric acid may be determined with phenoldisulphonic acid, but the brucine method does not give satisfactory results. Details are as follows: 1 ml. of a solution (10 to 100 mg./l.) in glacial acetic acid is treated with 2 ml. of phenoldisulphonic acid and heated for 10 minutes on the water-bath. After the addition of 25 ml. of ammonia solution (25 per cent.), the mixture is made up to 100 ml. The colour is determined photometrically (filter S43) using potassium nitrate as standard. For the determination of pentaerythrityl tetranitrate and glyceryl trinitrate in admixture in tablets the method is as follows: The sample is extracted with 3 ml. of acetone, and rinsed twice with 1 ml. of water. The filtered solution is made up to 100 ml. with water, and the pentaerythrityl tetranitrate which separates out is filtered off. The glyceryl trinitrate content of the solution is determined by the procaine-naphthylamine method, and the pentaerythrityl tetranitrate by difference from the total nitrate. G.M.

Persulphates, Iodimetric Titration of. D. J. de Jong. (*Pharm. Weekbl.*, 1948, 83, 596.) The reaction between persulphate and iodide is slow, but may be accelerated by traces of iron and by warming. The method recommended is as follows: 0.3 g. of the salt is dissolved in 20 ml. of water and treated with 3 g. of potassium iodide and 0.1 g. of ferrous sulphate, followed by 10 ml. of dilute hydrochloric acid and 50 ml. of water. The liberated iodine is titrated with 0.1N sodium thiosulphate until colourless, when starch solution is added, followed by 100 ml. of boiling water. The titration is then continued until the blue colour does not reappear. G.M.

Physostigmine, Photometric Determination of. I. Ehrlé. (*Farm. Revy.*, 1948, 47, 519.) The determination is based on the conversion of physostigmine to rubreserine by way of the following reactions:



The method has been used before, but in the present one somewhat different conditions are employed, with a more closely defined oxidation process. Rubreserine, gives similar, but not identical absorption curves in acid or alkaline solution, with two maxima, as follows:

at pH 1.10	460 mμ	log e = 3.56
	295 „	4.06
in 0.02 M sodium hydroxide	480 „	3.61
	298 „	4.11

At pH values 2 to 6, intermediate curves are obtained.

The pK_a value for rubreserine was found to be 4.03. The methods employed for the determination of physostigmine salicylate is as follows: 1 to 4 mg. is mixed with 1.0 ml. of a 2 per cent. solution of potassium ferricyanide, 1 ml. of M sodium hydroxide, and made up to 50 ml. with water. After 10 to 15 minutes the extinction is read at 480 mμ. A blank test is also carried out on the reagents. The molar extinction coefficient is between 3900 and 4000. G.M.

ESSENTIAL OILS

Cajuput Oil, Characters of. W. Spoon and W. M. Sessler. (*Pharm. Weekbl.*, 1948, 83, 593.) A sample of cajuput oil obtained from England showed the following characters; which are compared with the requirements of the Dutch Pharmacopœia:

	Sample	Dutch Pharmacopœia
Density 15°/15°C.	0.9150	0.919 to 0.930
Refractive index 20°C.	1.4698	1.466 to 1.471
Solubility in alcohol (80 per cent.)	in 1 to 10 vol.	in 1 vol.
[α] _D ...	-1.3°	—
Cineol (per cent.) ...	51.5	—
Distillate between 170° and 190°C.	59	66 per cent.

The sample was clear and colourless, or with at most a faint bluish tinge, and the odour was abnormal. Samples recently imported via Macassar also showed abnormal figures, as follows:

	Boeroe	Moluccas	Moluccas
Density 10°/15°C.	1.9145	0.9138	p.9126
Refractive index 20°C.	1.4691	1.4686	1.4674
[α] _D ...	-4.4°	-1.3°	-1.4°
Solubility in alcohol (80 per cent.) ...	1-10 vols	1-5-10 vols	1-10 vols
Cineol (per cent.) ...	59.6	60.4	60.4
Fatty Oils ...	absent	present	trace
Mineral oils ...	absent	absent	absent

These abnormal figures are apparently due to the difficulties of the reconstruction period in the producing areas. It would appear that the distillation is not carried as far as previously, so that the oil is deficient in heavy components. The presence of small quantities of coconut oil in two samples is also of interest.

G. M.

FIXED OILS, FATS AND WAXES

Fatty Acids, Saturated Straight-Chain, Separation of. L. L. Ramsey and W. L. Patterson. (*J. Ass. Off. agric. Chem.*, 1948, 31, 441.) The method given depends on partition chromatography and can be used for the straight-chain saturated fatty acids having 11 to 19 carbon atoms. A column of silicic acid is used with a mixture of furfuryl alcohol and 2-aminopyridine as the immobile solvent and *n*-hexane as the mobile solvent. Percolate fractions are titrated with standard sodium ethylate solution, while the separated acids are determined by titration in alcohol (70 per cent.) with standard sodium hydroxide. Each separated acid is tentatively identified by its threshold volume, confirmation being obtained by a melting-point determination or by the addition of an approximately equal amount of an authentic sample of the suspected acid to the unknown, and testing the homogeneity by chromatographic adsorption of the mixture on a fresh column. The separation of the even numbered acids from each other, and of the odd numbered members from each other, was fairly complete in a single fractionation and recoveries of added acids were essentially quantitative. Details of procedure are given, together with results obtained in investigating the purity of a number of fatty acids obtained from commercial sources.

R. E. S.

Neat's Foot Oil, Component Acids and Glycerides of. T. P. Hilditch and R. K. Shrivastava. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 139.) The component acids and glycerides of a sample of Irish neat's foot oil were determined by application of ester fractionation and of low temperature crystallisation from solvents. The acids were fractionated by crystallisation from ether followed by fractionation of their methyl esters. They consisted of myristic 0.7, palmitic 16.9, stearic 2.7, arachidic 0.1, tetradecenoic 1.2, hexadecenoic 9.4, oleic 64.4, octadecadienoic 2.3, octadecatrienoic 0.7, and unsaturated C_{20-22} acids 1.6 per cent. (wt.). The component glycerides, studied after partial separation by low-temperature crystallisation from acetone, were found to include about 35 per cent. of palmitodiolein, 23 per cent. of hexadecenodiolein, 8 per cent. of polyethenoid-diolein, 7 per cent. of oleopalmitostearin and probably not much more than 10 per cent. of triolein, with minor amounts of other mixed glycerides. The presence of fairly substantial proportions of hexadecenoic acid in neat's foot oil had not been previously noted. The specific utility of the oil as a lubricant cannot be connected with a high content of triolein, the present work suggesting the possibility that hexadecenodiolein (nearly one-quarter of the oil) and perhaps also the di-oleoglycerides in which the third acyl group is a polyethenoid member of the C_{18} , C_{20} (or C_{22}) series, may contribute specifically to its lubricant properties.

R. E. S.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Potato Amylose, Action of β -Amylase on. R. H. Hopkins, B. Jelinek and L. E. Harrison. (*Biochem. J.*, 1948, 43, 32.) The reaction between the purest amylose and β -amylase obtainable has been investigated with special attention to the reaction kinetics. The mixtures used in the investigations and the method for determining the progress of hydrolysis were as described by Hopkins *et al.* (*Biochem. J.*, 1946, 40, 507) except that in the presence of antiseptics or butyl alcohol the ferricyanide method of Cole was used. It was found that pure potato amylose was hydrolysed by β -amylase, at a steadily declining rate, to completion. Any "denaturation" of the amylose due to undue exposure to water caused a break in the progress curve and incomplete hydrolysis. Amylose crystallised from potato starch paste at pH 4, or from autoclaved paste, hydrolysed faster than from non-autoclaved pastes at pH 6 or 9. Relatively shorter molecular chains hydrolysed more slowly. Short-chain molecules (less than 12) were not present in amylose prepared by crystallisation methods according to evidence from iodine colours. The blue value of amylose increased with chain length approaching an asymptotic value although it decreased on hydrolysis. The reaction obeyed the formulation of Michaelis and Menten but the value of K_m varied widely with the preparation. A hypothesis is put forward to explain the observed kinetics.

R. E. S.

Potato Starch, Fractionation of. R. H. Hopkins and B. Jelinek. (*Biochem. J.*, 1948, 43, 28.) A number of methods were investigated with the object of obtaining the purest amylose and amylopectin. The processes of Schoch and of Haworth, Peat and Sagrott were used, and amylose and amylopectin were also prepared from non-autoclaved potato starch paste by successive additions on alternate days of cyclohexanol and thymol; the resulting products from the different methods did not show any great differences. Amylose produced under acid conditions showed a greater

tendency to retrograde in neutral solution. The action of hot water on amylose changed even the portion which does not retrograde, the blue value fell, and β -amylose action was incomplete. Preparations of amylose with 17 times the blue value of the corresponding amylopectin were obtained. Purification of amylopectin was less successful, but fractional ethyl alcohol precipitation removed some amylose.

R. E. S.

Potato Starch, Fractionation of. Part V. The Phosphorus of Potato Starch. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 121.) Fractions of potato starch, ground for differing periods in a ball mill, were examined for the distribution of phosphorus under various conditions. A small amount only of the phosphorus of potato starch was found to be present in dialysable form. Bound phosphorus was not split off from potato starch fractions by precipitation with alcohol and there were no significant differences between the bound phosphorus in the cold-water soluble and hot-water soluble potato starch fractions. The conclusion was reached that the phosphorus of potato starch is present, either largely or wholly, in the form of strongly-bound esterified phosphate groups, whilst in wheat starch, either all or a large proportion of the phosphorus is present in the form of less strongly-bound, adsorbed phosphatides. The work in the present paper coupled with that of other workers suggests that most of the phosphate groups in potato starch are bound to the amylopectin fraction, whereas in wheat starch the phosphatides are adsorbed, either largely or wholly, by the amylose fraction. The net result of this difference is to increase the hydrophilic character of potato starch solutions and pastes, but to decrease that of wheat starch solutions and pastes.

R. E. S.

Potato Starch, Fractionation of. Part VI. Retrogradation of Fractions. L. H. Lampitt, C. H. F. Fuller, N. Goldenberg and G. H. Green. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 179.) The literature concerning the retrogradation (changes in the physico-chemical state that take place in solutions, pastes, or gels on ageing) is briefly summarised and results are reported dealing with the retrograding properties of potato starch fractions as compared with wheat starch fractions. The various cold-water-soluble and hot-water-soluble fractions of starch obtained by grinding potato starch in a ball mill for varying periods were examined and the retrograding properties of the various fractions under varying conditions of temperature and concentration are reported; the properties were qualitatively similar to those of wheat starch fractions published earlier. The differences in retrograding properties of the various fractions depended on the contents of amylose and amylopectin and also on the differences in the nature and distribution of the small quantities of phosphorus present in the two starches.

R. E. S.

GUMS AND RESINS

Karaya Mucilage. K. E. Gronkvist. (*Farm. Revy*, 1948, 47, 623, 635.) Karaya gum has been introduced into the new Swedish Pharmacopœia under the name of Gummi sterculiæ. The viscosity of mucilages has been examined, using an Ostwald viscometer. The mucilages were prepared from coarsely powdered gum, and were passed through a homogeniser. When the mucilage was passed repeatedly through the capillary of the viscometer, the apparent viscosity at first decreased, then rose to a constant value. The same pheno-

menon was shown by an alkaline solution, but on ageing the viscosity decreased and became less variable. The maximum viscosity is obtained at pH 5 to 7, and decreases sharply on the alkaline side. In all cases of fully hydrated mucilages, the viscosity decreases on keeping, but the decrease is more rapid with a high pH value. Heating the dry gum causes the hydration of the mucilage to be very slow, so that such mucilages show a rise in viscosity over a period of some months. The pH of the mucilage is not affected by heat, but decreases on storage.

G. M.

ORGANIC CHEMISTRY

β -Peltatin, A New Compound from Podophyllin. J. L. Hartwell and W. E. Detty. (*J. Amer. chem. Soc.*, 1948, 70, 2833.) The fractionation of podophyllin by chromatographic adsorption has yielded 4 per cent. of a new crystalline substance for which the name β -peltatin is proposed. The new compound possesses about the same high necrotising activity for mouse sarcoma 37 as α -peltatin. β -Peltatin crystallises from alcohol in colourless, transparent prisms, m.pt. 231.1 to 238.0°C. (shrinks at 225.5°C.) cor.; $[\alpha]_D^{20}$ -115° (c, 1.009 absolute alcohol). Analysis showed a formula $C_{22}H_{22}O_8$ with three methoxy groups. Molecular weight values (Rast) for derivatives of both α - and β -peltatin agree with the formula $C_{22}H_{22}O_8$ and indicate that the peltatins are thus isomeric with podophyllotoxin. α -Peltatin has one methoxy group less than β -peltatin and podophyllotoxin. Both α - and β -peltatins give an immediate yellow colour with sulphuric acid but with α -peltatin the colour turns reddish-brown, with β -peltatin green, before becoming finally red.

R. E. S.

Sodium Citrate, Water of Crystallisation of. T. A. G. Haanappel. (*Pharm. Weekbl.*, 1948, 83, 687.) The Dutch Pharmacopœia describes crystalline sodium citrate with 5 H₂O. Actually the highest hydrated salt contains 5½ H₂O. The Pharmacopœia also states that the salt effloresces at a relative humidity less than 0.3; investigation showed that this figure should be 0.6.

G. M.

Stilbamidine: Instability of. A. J. Henry. (*Brit. J. Pharmacol.*, 1948, 3, 163.) Hydrolysis of the amidine groups of stilbamidine is a dark reaction. Examination of solution of stilbamidine after storage for three years under various conditions shows that the factors on which the rate of hydrolysis of the amidine groups primarily depends are the pH of the solution and the temperature. Storage in the dark at 40°C. of a 1 per cent. solution of the hydrochloride at pH 7 will almost certainly produce a good crop of crystals of *trans*-4-amido-4'-amidinostilbene hydrochloride within three months. A pH of 5 suppresses hydrolysis almost indefinitely. *Trans*-4-amido-4'-amidinostilbene is more toxic than the parent compound. Its formation in the body—for which conditions of pH and temperature would be favourable—from stilbamidine absorbed and retained for long periods may, therefore, in part account for the delayed toxic effects which have been observed. The occurrence of such prolonged storage in the body is supported by recent examination of the urine of kala-azar patients some 18 months after termination of a course of treatment; stilbamidine, or a closely related derivative, is still being excreted at a low level (0.005 to 0.03 mg/100 ml.). It seems highly probable that similar conditions of adsorption, storage and slow release apply for the dimer after administration as appear to apply for stilbamidine.

S. L. W.

PLANT ANALYSIS

Alkaloids, Isolation of, From Plants. N. Lörgren. (*Svensk Farm. Tidskr.*, 1949, 53, 1.) The dried material is first extracted with an organic solvent containing a small quantity of an organic acid which forms insoluble salts with the alkaloids. The alkaloids are then extracted with an organic solvent containing dry ammonia gas. As applied to *Chelidonium majus*, details are as follows. 75 g. of the root is treated with 230 ml. of anhydrous ether containing 2 g. of anhydrous oxalic acid. After shaking for 3 hours, the mixture is filtered and the residue is treated with a further quantity of 150 ml. of ether and 0.5 g. of oxalic acid. After filtering again, the residue is suspended in ether, dry ammonia gas is passed in, and after standing overnight, the extraction is continued in a continuous extraction apparatus for 2 days. The solution is filtered and the ether distilled off. The residue is dissolved in a mixture of 15 ml. of chloroform and 15 ml. of dry ether, then treated with 100 ml. of a saturated solution of anhydrous citric acid in ether. The precipitate is filtered off and dried *in vacuo*. For ergot the defatted drug is extracted exhaustively with ethyl acetate containing ammonia gas. The solution is concentrated at a low temperature, and the alkaloids are precipitated by the addition of a saturated solution of citric acid in ether. The precipitate is allowed to settle, separated by centrifuging, washed with light petroleum, and dried *in vacuo*. It contains all the alkaloids of the drug in very good yield. G. M.

Cascara Sagrada, Chromatographic Isolation of Trihydroxymethylanthraquinones. M. R. Gibson and A. E. Schwarting. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 206.) Chloroformic extracts of extract of cascara sagrada, U.S.P., were run through a 30-mm. tube of celite, 3 parts, and magnesia, 1 part. The red-brown, deep red, orange and pink layers obtained were separated, treated with 10 per cent. hydrochloric acid to dissolve the magnesia and shaken out with chloroform. When the chloroformic solutions were run through 12 mm. tubes of the same adsorbent, the red-brown layer proved to be a mixture of the anthraquinones, whereas the deep red, orange and pink layers were homogeneous, and contained the three anthraquinones (emodin, aloe-emodin and isoemodin) each contaminated with an unknown substance, which could be removed by subliming under reduced pressure. The progress of the separation and purification was followed by similar experiments on various mixtures of the pure anthraquinones, and by spectrophotometric measurements. The method is not suitable for quantitative isolation of the anthraquinones unless the nature of impurities appearing in the first resins is known. A pale yellow layer which appeared immediately below the adsorbed anthraquinones in all experiments was not identified but was shown not to interfere appreciably with the spectrophotometric measurements over the range of wave-lengths used. The use of spectrophotometry showed its suitability as a method for the quantitative analysis of mixtures of the anthraquinones. The method described for the preparation of pure isoemodin was a modification of that of Green, King and Beal (*J. Amer. pharm. Ass.*, 1938, 27, 95) and gave bright orange crystals melting at 179°C.

G. R. K.

Pyrethrin Content of *Chrysanthemum cinerariæfolium* flowers. M. G. Edwards. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 379.) The pyrethrins were rapidly extracted from the undried flowers by grinding 400 g. with 120 g. of light petroleum (55° to 60°C.) in a rod mill for 2 hours. A further 500

g. of light petroleum was added, grinding continued for 2 minutes and the marc washed until the washings were colourless. In this way some 95 per cent. of the pyrethrins were extracted; the remainder can be obtained by a 7-days' maceration or in a Soxhlet. Three strains of flowers were investigated—(a) large flowers of low pyrethrin content; (b) "high toxic" flowers; (c) commercial Grade 1 flowers. To obtain a comparison with moisture-free flowers, samples were dried (a) in a draught at room temperature, for 12 to 14 days; (b) in a current of air at 55°C., for 7 to 8 hours; (c) in a commercial drying tunnel at 55°C., for 24 hours. The main extract was concentrated and the pyrethrins determined in aliquot portions by the A.O.A.C. method, modified by the use of hydrochloric acid in place of sulphuric acid to acidify before the light petroleum extraction of the monocarboxylic acid. The free chrysanthemum acids were not extracted and are included in the pyrethrins. Tables show the pyrethrins in the extracts of fresh flowers, and corresponding dried flowers, calculated to moisture-free basis. The results indicate that the undried flowers contain about the same amount of pyrethrin I, and about 10 per cent. more of pyrethrin II than the same flowers dried in the most favourable way, and 3 to 4 per cent. more pyrethrin I and 12 to 13 per cent. more pyrethrin II than flowers dried by one particular commercial method.

H. F.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Antipyretic Action and Catalase Activity. J. Williamson and E. A. Rudge. (*Biochem. J.*, 1948, 43, 15.) Many antipyretics can be used to stabilise hydrogen peroxide solution and several representative antipyretic substances were therefore tested for inhibitory activity on the isolated catalase-hydrogen peroxide system in an attempt to provide evidence for the hypothetical suggestion that such substances lower the temperature of the body by reducing the rate of oxidation, conceivably by interfering with hydrogen peroxide metabolism. Of the substances tested, acetanilide and phenacetin represented typical aniline derivatives, salicylic acid and acetylsalicylic acid represented typical phenol derivatives, phenazone represented pyrazolone derivatives, and quinine represented quinoline derivatives; *p*-hydroxybenzoic acid was also examined. Parallelism with antipyretic activity was not found since inhibition of the *in vitro* catalase-hydrogen peroxide system was observed with some, but not all, of the substances tested. A horse liver catalase extract was used: phenacetin, phenazone and quinine showed no inhibition; acetanilide showed a slight inhibition, the greatest inhibitory action being obtained with salicylic acid, acetylsalicylic acid and *p*-hydroxybenzoic acid. It is suggested that the observed inhibitions with the hydroxybenzoic acid derivatives result from the presence of phenolic -OH groupings.

R. E. S.

Bacitracin. Production and Properties of Crude Substance. H. S. Anker, B. A. Johnson, J. Goldberg and F. L. Meloney. (*J. Bact.*, 1948, 55, 249.) Bacitracin, which was first extracted from a culture of an organism of the *Bacillus subtilis* group isolated from an infected wound, has not yet been obtained in the pure state. An arbitrary unit has been defined as the amount which when diluted 1 in 1024 completely inhibits the growth of a stock strain of group A hæmolytic streptococcus. It can be assayed by a serial dilution method, or by a plate method using

strains of *Corynebacterium*. * Suitable media for production purposes are an *l*-glutamic acid synthetic medium or a soya bean digest medium. Extraction cannot be effected by a number of common solvents but *n*-butanol extracts 85 to 90 per cent. of the activity. The butanol is distilled from the extract in the presence of water, giving a 100-fold concentration. Purification is effected by butanol-ether extraction at pH 3 to 4, the activity remaining in the aqueous layer. The latter is extracted with ether, distilled under reduced pressure, and freeze-dried after neutralisation. An alternative method of purification using magnesium oxide with subsequent precipitation as the salicylate is also described. Aqueous solutions of crude bacitracin are stable at 0° to 5°C. for 8 to 12 months, but at higher temperatures there is a loss of activity which is complete at 37°C. in two weeks. Alkali causes rapid inactivation; so also does hydrogen peroxide, but thiol compounds cause no measurable change. Thioglycollic acid partially reactivates peroxide-inactivated material. The substance is soluble in alcohols but insoluble in many other organic solvents. Diffusion experiments indicate a molecular weight below 2000. It is precipitated by heavy metals and by several organic acids, sometimes, for example, by salicylic acid, without loss of activity. Chemical examination shows that the substance is not a peptide, and that it lacks guanido and phenolic groups.

H. T. B.

Diphtheria Toxoid: Improvement in Preparation. L. B. Holt. (*Brit. J. Exp. Path.*, 1948, 29, 343.) Diphtheria toxoid produced in the ordinary way is first treated with magnesium hydroxide to remove colour, inorganic phosphates, and some protein. It is then treated with cadmium chloride solution and finally fractionated with ammonium sulphate.

H. T. B.

Liver Extract, Purified; Chemical Nature as Determined by Paper Partition Chromatography. G. H. Tishkoff, A. Zaffaroni and H. Tesluk. (*J. biol. Chem.*, 1948, 175, 857.) Results are given for the investigation of a commercial, highly purified, liver extract containing the antipernicious anaemia factor using two-dimensional paper partition chromatography. The presence of one or more polypeptides of high molecular weight was proved; the polypeptide material was separated and the free amino acids were liberated from it by hydrolysis. The following amino-acids were obtained: leucine, glycine, alanine, aspartic acid, valine, proline (relatively large amounts); arginine, lysine, glutamic acid; serine, phenylalanine, threonine, hydroxyproline (medium amounts); histidine, cysteine, methionine (small amounts). Some free amino acids were found in the original preparation and also riboflavine, but folic acid and xanthopterin were not found.

R. E. S.

Penicillin, Radioactive, Studies with. D. Rowley, J. Miller, S. Rowlands and E. Lester Smith. (*Nature*, 1948, 161, 1009.) In view of the possibility that penicillin may act by depriving sensitive organisms of glutamic acid, which is probably essential to their growth, the authors have attempted to ascertain whether penicillin, by being itself absorbed, blocks the passage of glutamic acid through the cell wall. As the amount of penicillin taken up by organisms is too small to detect by biological methods, it was decided to attempt its detection by using radioactive penicillin of a high specific activity, namely, 0.05 microcurie per I.U., the radioactivity of the penicillin being measured by means of a Geiger-Müller counter having a background of 14 counts per minute. For the absorption experiments, *Staphylococcus aureus* was grown on agar plates

g. of light petroleum was added, grinding continued for 2 minutes and the marc washed until the washings were colourless. In this way some 95 per cent. of the pyrethrins were extracted; the remainder can be obtained by a 7-days' maceration or in a Soxhlet. Three strains of flowers were investigated—(a) large flowers of low pyrethrin content; (b) "high toxic" flowers; (c) commercial Grade 1 flowers. To obtain a comparison with moisture-free flowers, samples were dried (a) in a draught at room temperature, for 12 to 14 days; (b) in a current of air at 55°C., for 7 to 8 hours; (c) in a commercial drying tunnel at 55°C., for 24 hours. The main extract was concentrated and the pyrethrins determined in aliquot portions by the A.O.A.C. method, modified by the use of hydrochloric acid in place of sulphuric acid to acidify before the light petroleum extraction of the monocarboxylic acid. The free chrysanthemum acids were not extracted and are included in the pyrethrins. Tables show the pyrethrins in the extracts of fresh flowers, and corresponding dried flowers, calculated to moisture-free basis. The results indicate that the undried flowers contain about the same amount of pyrethrin I, and about 10 per cent. more of pyrethrin II than the same flowers dried in the most favourable way, and 3 to 4 per cent. more pyrethrin I and 12 to 13 per cent. more pyrethrin II than flowers dried by one particular commercial method.

H. F.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Antipyretic Action and Catalase Activity. J. Williamson and E. A. Rudge. (*Biochem. J.*, 1948, 43, 15.) Many antipyretics can be used to stabilise hydrogen peroxide solution and several representative antipyretic substances were therefore tested for inhibitory activity on the isolated catalase-hydrogen peroxide system in an attempt to provide evidence for the hypothetical suggestion that such substances lower the temperature of the body by reducing the rate of oxidation, conceivably by interfering with hydrogen peroxide metabolism. Of the substances tested, acetanilide and phenacetin represented typical aniline derivatives, salicylic acid and acetylsalicylic acid represented typical phenol derivatives, phenazone represented pyrazolone derivatives, and quinine represented quinoline derivatives; *p*-hydroxybenzoic acid was also examined. Parallelism with antipyretic activity was not found since inhibition of the *in vitro* catalase-hydrogen peroxide system was observed with some, but not all, of the substances tested. A horse liver catalase extract was used; phenacetin, phenazone and quinine showed no inhibition; acetanilide showed a slight inhibition, the greatest inhibitory action being obtained with salicylic acid, acetylsalicylic acid and *p*-hydroxybenzoic acid. It is suggested that the observed inhibitions with the hydroxybenzoic acid derivatives result from the presence of phenolic -OH groupings.

R. E. S.

Bacitracin. Production and Properties of Crude Substance. H. S. Anker, B. A. Johnson, J. Goldberg and F. L. Meleney. (*J. Bact.*, 1948, 55, 249.) Bacitracin, which was first extracted from a culture of an organism of the *Bacillus subtilis* group isolated from an infected wound, has not yet been obtained in the pure state. An arbitrary unit has been defined as the amount which when diluted 1 in 1024 completely inhibits the growth of a stock strain of group A haemolytic streptococcus. It can be assayed by a serial dilution method.

for the determination of barbiturates in blood and tissues based on the characteristic ultraviolet absorption spectra of the malonylurea ring structure. The drug is extracted by an organic solvent, usually chloroform, and then re-extracted with alkali. The ultraviolet absorption spectra of the alkaline solution is determined against a reference blank solution of sodium hydroxide using the photoelectric quartz spectrophotometer. Absorption spectra of some representative barbiturates, e.g., seconal and amytal, showed an intense ultraviolet absorption with a maximum at $225m\mu$ and a minimum of $235m\mu$. At $255 m\mu$ the concentration bears a linear relationship to the optical density up to at least $20 \mu\text{g./ml.}$ for these barbiturates. The method is sensitive to 0.4 mg./100 ml. of blood and 1.0 mg./100 g. of tissue, with an error of less than 10 per cent.

E. N. I.

Heparin, Assay of. C. N. Mangieri. (*J. Lab. clin. Med.*, 1947, 32, 901.) Fresh bovine blood containing 50 ml. of 8 per cent. solution of sodium citrate per l. is used. The plasma may be used fresh or after storage at -20°C. with equal accuracy. It is recalcified before assay with a predetermined amount of calcium chloride dissolved in 0.2 ml. of physiological saline solution so that the end-point for clotting lies near the middle of the series of twelve tubes. Two series of tubes are set up, one containing the standard and the other, the unknown heparin. $10 \mu\text{g./ml.}$ of standard or purified heparin or 20 to $40 \mu\text{g.}$ of crude heparin is used. The standard is diluted to 1.0 Toronto unit/ml. The first tube of each series contains 0.23 ml. of heparin solution and this is increased by 0.02 ml. up the series. The volume of every tube is then made up to 0.8 ml. with saline solution. To the first tube of each series 1.0 ml. of plasma and 0.2 ml. of the calcium chloride solution are added. The contents are gently mixed and incubated at 37°C. for 3 hours. This is repeated with each 2 corresponding tubes in the series at a time. The end-point is the tube in which clotting is just prevented after 3 hours. Fluorescent light is recommended for detecting thin films of clot. The activity of the heparin under test is obtained from the quantities of heparin in each of the end-point tubes. The author claims that the end-point is definite and that results are reproducible with less than 10 per cent. of error.

A. D. O.

Theophylline in Blood and Urine, Determination of. A. J. Plummer. (*J. Pharmacol.*, 1948, 93, 142.) To 4 ml. of a methyl alcohol solution of theophylline in a 15-ml. centrifuge tube add 5 ml. of a saturated solution of copper acetate, and allow to stand tightly-stoppered for 4 hours to ensure complete precipitation of the theophylline-copper compound; centrifuge for 15 minutes at 1000 r.p.m.; decant the supernatant fluid, drain, and wash the precipitate with 5 ml. of methyl alcohol; again centrifuge, decant, and drain; dissolve the precipitate in 4 ml. of 0.2N sulphuric acid and add 0.5 ml. of potassium iodide solution (1 g. to 1 ml. of water); titrate the liberated iodine with 0.02N sodium thiosulphate, using soluble starch solution as indicator; each mg. of theophylline is equivalent to 0.57 ml. of 0.02N sodium thiosulphate. For blood, deproteinise by adding 25 parts of blood to 40 parts of 13 per cent. trichloroacetic acid; allow to stand 20 minutes; filter or centrifuge; render just alkaline to litmus with 2.5N sodium hydroxide; add 10 ml. of a phosphate buffer of pH 8.0, the final pH must be between 7.3 and 8.2; extract the theophylline by shaking the buffered filtrate with three 20-ml. quantities of a mixture of 3 volumes of chloroform and 1 volume of isopropyl alcohol for 5 minutes each extraction; evaporate the combined extracts just to dryness on a water-bath;

at 37°C. for 9 hours, scraped off, transferred to 25 ml. of broth, so as to give a thick suspension, and incubated for a further 2 hours. A measured amount of radioactive penicillin was then added and an aliquot portion of the suspension removed for radioactive assay, this removal of a standard volume of the suspension being repeated at intervals as a control. After $\frac{1}{2}$, 2, 6 and 24 hours, 5 ml. of the suspension was removed and filtered through a "Gradocol" membrane, and washed through with 2 ml. of water into a graduated receiver. Aliquot portions of these filtrates were taken, dried, weighed and counted. Any uptake of penicillin by the bacteria should be shown by a decrease in penicillin concentration in the filtrate. From the results of an experiment in which 0.07 I.U. per ml. was employed in a suspension containing 3.2×10^7 viable organisms per ml., it was seen that the greatest difference in the penicillin concentrations in the filtrates was no greater than that for the suspensions. Corresponding results were obtained from other experiments, using penicillin concentrations of from 1 down to 0.05 I.U./ml. From these experiments the authors conclude that absorption of penicillin, if any, probably amounts to less than 10 molecules per bacterium.

S. L. W.

Pyridine Nucleotides, Extinction Coefficients of the Reduced Band of. B. L. Horecker and A. Kornberg. (*J. biol. Chem.*, 1948, 175, 385.) Precise values for the extinction coefficients of diphosphopyridine nucleotide and triphosphopyridine nucleotide were determined on partly pure preparations by the use of pure substrates in reactions which are essentially complete. Such determinations have been made with pyruvic acid, acetaldehyde and isocitric acid. The systems actually used were pyruvate di- and tri-phosphopyridine nucleotide, isocitrate-triphosphopyridine nucleotide, and acetaldehyde-diphosphopyridine nucleotide. A molecular extinction coefficient of 6.22×10^6 for the reduced forms of both di- and tri-phosphopyridine nucleotides at 340m μ . was obtained.

R. E. S.

BIOCHEMICAL ANALYSIS

Ascorbic Acid in Food Preparations, Estimation of. S. A. Goldblith and R. S. Harris. (*Anal. Chem.*, 1948, 20, 649.) It is shown that both the indophenol method and the method based on coupling with 2:4-dinitrophenylhydrazine give satisfactory results in estimating the ascorbic acid content of fresh vegetables. The indophenol technique measures biologically active ascorbic acid while the dinitrophenylhydrazine method measures 1-ascorbic acid, dihydroascorbic acid and 2:3-diketogulonic acid. The ascorbic acid in oxalated slurries (with four parts of 0.5 per cent. oxalic acid) was oxidised almost completely to dehydroascorbic acid in 21 days while the values obtained by the dinitrophenylhydrazine method remained constant for 14 days. It is claimed that inasmuch as the indophenol and dinitrophenylhydrazine methods agree when used to measure ascorbic acid in ground-fresh plant materials and disagree more and more during storage after the plant is taken from the ground, these methods should prove useful in checking the freshness of perishable vegetables. The ascorbic acid content of garden-fresh edible plants may thus be measured in a laboratory remote from the harvest area. Both methods may be employed to establish the freshness of vegetable foods.

R. E. S.

Barbiturates in Blood and Tissues, An Ultraviolet Spectrophotometric Procedure for the Determination of. L. R. Goldbaum. (*J. Pharmacol.*, 1948, 94, 68.) A simple, rapid and highly specific procedure is described

for the determination of barbiturates in blood and tissues based on the characteristic ultraviolet absorption spectra of the malonylurea ring structure. The drug is extracted by an organic solvent, usually chloroform, and then re-extracted with alkali. The ultraviolet absorption spectra of the alkaline solution is determined against a reference blank solution of sodium hydroxide using the photoelectric quartz spectrophotometer. Absorption spectra of some representative barbiturates, e.g., seconal and amytal, showed an intense ultraviolet absorption with a maximum at $225m\mu$ and a minimum of $235m\mu$. At $255 m\mu$ the concentration bears a linear relationship to the optical density up to at least $20 \mu\text{g./ml.}$ for these barbiturates. The method is sensitive to 0.4 mg./100 ml. of blood and 1.0 mg./100 g. of tissue, with an error of less than 10 per cent.

E. N. I.

Heparin, Assay of. C. N. Mangieri. (*J. Lab. clin. Med.*, 1947, 32, 901.) Fresh bovine blood containing 50 ml. of 8 per cent. solution of sodium citrate per l. is used. The plasma may be used fresh or after storage at -20°C. with equal accuracy. It is recalcified before assay with a predetermined amount of calcium chloride dissolved in 0.2 ml. of physiological saline solution so that the end-point for clotting lies near the middle of the series of twelve tubes. Two series of tubes are set up, one containing the standard and the other, the unknown heparin. $10 \mu\text{g./ml.}$ of standard or purified heparin or 20 to $40 \mu\text{g.}$ of crude heparin is used. The standard is diluted to 1.0 Toronto unit/ml. The first tube of each series contains 0.23 ml. of heparin solution and this is increased by 0.02 ml. up the series. The volume of every tube is then made up to 0.8 ml. with saline solution. To the first tube of each series 1.0 ml. of plasma and 0.2 ml. of the calcium chloride solution are added. The contents are gently mixed and incubated at 37°C. for 3 hours. This is repeated with each 2 corresponding tubes in the series at a time. The end-point is the tube in which clotting is just prevented after 3 hours. Fluorescent light is recommended for detecting thin films of clot. The activity of the heparin under test is obtained from the quantities of heparin in each of the end-point tubes. The author claims that the end-point is definite and that results are reproducible with less than 10 per cent. of error.

A. D. O.

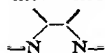
Theophylline in Blood and Urine, Determination of. A. J. Plummer. (*J. Pharmacol.*, 1948, 93, 142.) To 4 ml. of a methyl alcohol solution of theophylline in a 15-ml. centrifuge tube add 5 ml. of a saturated solution of copper acetate, and allow to stand tightly-stoppered for 4 hours to ensure complete precipitation of the theophylline-copper compound; centrifuge for 15 minutes at 1000 r.p.m.; decant the supernatant fluid, drain, and wash the precipitate with 5 ml. of methyl alcohol; again centrifuge, decant, and drain; dissolve the precipitate in 4 ml. of 0.2N sulphuric acid and add 0.5 ml. of potassium iodide solution (1 g. to 1 ml. of water); titrate the liberated iodine with 0.02N sodium thiosulphate, using soluble starch solution as indicator; each mg. of theophylline is equivalent to 0.57 ml. of 0.02N sodium thiosulphate. For blood, deproteinise by adding 25 parts of blood to 40 parts of 13 per cent. trichloroacetic acid; allow to stand 20 minutes; filter or centrifuge; render just alkaline to litmus with 2.5N sodium hydroxide; add 10 ml. of a phosphate buffer of pH 8.0, the final pH must be between 7.3 and 8.2; extract the theophylline by shaking the buffered filtrate with three 20-ml. quantities of a mixture of 3 volumes of chloroform and 1 volume of isopropyl alcohol for 5 minutes each extraction; evaporate the combined extracts just to dryness on a water-bath:

dissolve the residue in methyl alcohol; transfer the solution to a 15-ml. graduated centrifuge tube, keeping the final volume of methyl alcohol between 0.3 and 0.5 ml.; determine as previously described, using 0.005N sodium thiosulphate solution for the final titration; each mg. of theophylline is equivalent to 2.28 ml. of 0.005N sodium thiosulphate. For urine, adjust the pH of the urine to between 7.3 and 8.2, and continue with the determination as for blood, commencing with the words "extract the theophylline . . ."; the volume of methyl alcohol used to dissolve the theophylline should be from 1 to 2 ml., and 0.01N sodium thiosulphate should be used for the final titration; each mg. of theophylline is equivalent to 1.14 ml. of 0.01N sodium thiosulphate. The method is sensitive to 0.13 mg. of anhydrous theophylline per 100 ml. of blood or urine. From 15 to 20 ml. samples of blood are satisfactory. A dilute urine may be used directly, but concentrated urine should be diluted with 3 or 4 volumes of water. Water should be rigidly excluded from the solutions when precipitating the theophylline with copper acetate, or the precipitation may not be quantitative. Caffeine, theobromine, uric acid, ethylenediamine and sodium acetate do not interfere with the determination, nor does any normal blood property or constituent.

S. L. W.

CHEMOTHERAPY

Anthelmintic Potency in Relation to Chemical Constitution. E. Baldwin. (*Brit. J. Pharmacol.*, 1948, 3, 91.) A report on the results of tests carried out *in vitro* on over 200 chemical compounds for the detection of anthelmintic potency, using an *Ascaris* preparation of which the muscle is directly exposed to the action of the drug. Significant activity was found among aliphatic-aromatic and aromatic-aromatic ketones, but nothing approaching the activity of santonin was discovered in this group. Considerable activity was observed among lactones, but here again none approached the activity of santonin. These facts seem to support the suggestion that the outstanding anthelmintic efficacy of santonin is due to the simultaneous presence of both ketonic and lactonic groups in its structure, rather than to either alone. Among the lactones, phenols and pyridines tested it was noted that anthelmintic activity increased with the addition of a second (usually a benzene) ring to the parent molecule and that activity was greater when the two rings were independent than when they were fused. The value of phenolic carbamates was confirmed, and an unusually high order of potency demonstrated in 2-hydroxydiphenyl carbamate. None of the thiazoles examined showed much promise of useful potency, but among the pyridines an outstanding order of activity was shown by 4-benzylpyridine, and more especially by 2-2'-dipyridyl and 4:5-phenanthroline. The



linkage in the last two compounds and in the corresponding tripyridyl possesses properties which seem to offer considerable possibilities in the search for new and highly efficacious anthelmintics. No activity was discovered among a number of microbial antibiotics, and there is no reason to think that penicillin, or the sulphonamides can yield new anthelmintic agents of any practical value. The importance of using experimental material of nematode origin as the basis of methods of this kind is strongly emphasised.

S. L. W.

Antimalarial Compounds. Studies in the Chemotherapy of Tuberculosis. E. Hoggarth and A. R. Martin. (*Brit. J. Pharmacol.*, 1948, 3, 156.)

CHEMOTHERAPY

Antituberculous activity in mice has been demonstrated with a new group of compounds, 2-aryl-amino-4-dialkylamino-6-methyl-pyrimidines, some members of which are active as antimalarial drugs. The compound showing most promise was 2-*p*-chloroanilino-4- δ -diethylamino- α -methylbutylamino-6-methylpyrimidine dihydrochloride (No. 3300). No activity was found with the other antimalarial drugs tested, including quinine, mepacrine and pamaquin. S. L. W.

Diaminomethylpyrimidines and Related Compounds: Studies in the Chemotherapy of Tuberculosis. E. Hoggarth, A. R. Martin, M. F. C. Paige, M. Scott and E. Young. (*Brit. J. Pharmacol.*, 1948, 3, 160.) More than 100 diaminomethylpyrimidines and related compounds have been examined for antituberculous activity in mice. The aim of the investigation was to discover a compound with greater activity than that possessed by No. 3300 (*Brit. J. Pharmacol.*, 1948, 3, 156). This aim was not realised, and it would appear that in compound No. 3300 itself and a number of closely related compounds the maximum activity possible in this particular chemical group has been reached. S. L. W.

Sulphonamides: Studies in the Chemotherapy of Tuberculosis. E. Hoggarth, A. R. Martin and E. H. P. Young. (*Brit. J. Pharmacol.*, 1948, 3, 153.) The activity of a series of 2-sulphanilamido-4:6-dialkoxy-pyrimidines and some closely related compounds against *M. tuberculosis* *in vitro* has been studied. In the former group, activity *in vitro* increases with increasing size of the alkoxy groups, but activity is limited by the consideration that substituents sufficiently large to confer high activity *in vitro* result in such poor adsorption that activity *in vivo* cannot be expected. The *di-n*-propoxy and *di-isopropoxy* compounds produced a significant increase in the mean survival times of groups of mice infected with *M. tuberculosis* when the drug was given both before and after the mice were infected, but when drug treatment was delayed for 24 hours no therapeutic effect was demonstrated. The higher members of the series were very poorly absorbed and failed to show any therapeutic action. S. L. W.

PHARMACY

DISPENSING

Sodium Citrate, Sodium Chloride and Glucose, Preparation of Sterile Solutions of. P. G. Horweg and G. V. D. Reyden. (*Pharm. Weekbl.*, 1948, 83, 684.) A solution containing 3.3 per cent. of sodium citrate, 5 per cent. of glucose, and 0.9 per cent. of sodium chloride was found to develop a turbidity on storing, especially under tropical conditions. The deposit consisted of silicates, although a specially hard glass was used for the containers. The precautions which were found necessary to prevent this precipitation were found to be the following: use of special hard glass; closures of a suitable plastic, and not of rubber; washing the asbestos filter, used to remove pyrogen, with a very large quantity of distilled water before use. G. M.

Sulphathiazole for Injection, Sterilisation of. P. Morch. (*Arch. Pharm. Chemi.*, 1948, 55, 575.) Sulphathiazole dissolves in alkali with a yellow colour, which is increased on heating. In alkaline solutions the compound is actually present as a resonance form and hydrolysis may occur. The author

ABSTRACTS

describes a method for determining 3 of the products as a measure of the degree of decomposition. Results are summarised thus:

Atmosphere in ampoule			Heat treatment	Decomposed per cent.		
Air	1 hour at 100°C.	0.04
Air	20 minutes at 120°C.	0.08
Air	1 hour at 120°C.	0.28
Nitrogen	20 minutes at 120°C.	0.05
Oxygen	20 minutes at 120°C.	0.12

The discoloration of the solutions was proportional to the amount of decomposition. The preparation is very sensitive to light: after standing in sunlight for three weeks the solution became dark red and had decomposed to the extent of about 3 per cent. The content of sulphathiazole was not appreciably changed by heat treatment. It is concluded that sterilisation of a sulphathiazole solution (pH about 9.5) should be carried out at 120°C. for 20 minutes.

G. M.

GALENICAL PHARMACY

Morphine and Apomorphine, Stability of Solutions of. A. Ionescu-Matiu, A. Popescu and L. Monciu. (*Ann. pharm. Fr.*, 1948, 6, 137.) The degree of decomposition of a solution of morphine or apomorphine may be determined by determining the ferricyanide value, as follows. To 1 to 5 ml. of a 1 per cent. solution of morphine 4 ml. of ferricyanide reagent (4 per cent. of potassium ferricyanide with 4 per cent. of potassium hydroxide), and 20 ml. of water are added. After boiling for 5 minutes and cooling, 50 ml. of water is added, followed by 5 ml. of sulphuric acid (20 per cent.). The mixture is then titrated with permanganate until a pink colour persists for 1 minute. By using this reaction, it was shown that a solution for injection of morphine hydrochloride had undergone from 2 to 12 per cent. of decomposition in 1 year, according to the conditions under which it was kept. The decomposition may be prevented by the addition of sulphite according to the following formula: morphine hydrochloride 0.40 g., sodium bisulphite solution (10 per cent.) 1 drop, water to 10 ml. Sterilisation is for 20 minutes at 100°C. A corresponding formula is used for apomorphine. In tinctures and other liquid galenical preparations, the ferricyanide process may be applied to the extracted morphine. The results showed that the degree of decomposition in one year did not exceed 15 per cent. It is recommended that these preparations should be stabilised with sulphite or benzoic acid.

G. M.

PHARMACOGNOSY

Atropa Belladonna, Frequency Determinations of. D. D. Boswijk. (*Pharm. Weekbl.*, 1948, 83, 609.) Frequency determinations of *Atropa Belladonna* have now been extended to 6 further samples from different localities. The results in general agree with those found previously (*Pharm. Weekbl.*, 1948, 83, 225; *Quart. J. Pharm. Pharmacol.*, 1948, 21, 534), but a sample from Leyden showed an abnormal frequency for the stomata in the upper epidermis, ranging from 63 to 88. In samples from Groningen, Delft and Leyden, trichomes with unicellular stalks and multicellular glands were prominent; while multicellular stalks and unicellular glands were general in *Atropa Belladonna* var. *lutea*.

G. M.

Cinchona Ledgeriana Bark. Distribution and Interrelationships of Alkaloids in. H. F. Birch and L. R. Doughty. (*Biochem. J.*, 1948, 43, 38.) The distribution of alkaloids throughout the whole bark of 3 trees of *Cinchona Ledgeriana*, each 7 years old, was investigated. Two of the trees had suffered damage at an earlier stage of their growth and differed markedly in shape from the third tree which was of apparently normal growth and straight in form. Samples of bark were analysed for total alkaloids, quinine, cinchonidine, and for amorphous alkaloids. The distribution of alkaloids in the stem bark was a function of bark thickness which is at any point inversely proportional to the distance of that point from the base of the tree. This basic regular decrease in alkaloid content from the base of the tree upwards was interrupted by zones of relatively high alkaloid content due to local thickness of the bark where the main stem forked, usually caused by damage of the stem at an earlier stage in the tree's life, where secondary leaders arose and where large branches joined the stem. Significant increases in alkaloidal content associated with unit increase in bark weight were found for total alkaloids, quinine, and cinchonidine/unit increase in bark weight, and for quinine, cinchonidine, and cinchonine/unit increase in total alkaloids. These increases were large compared with the small and insignificant increases (and decreases in the case of one tree) found for the amorphous alkaloids, an indication of the progressive conversion of amorphous to crystallisable alkaloids during the life of the tree. When the trees were felled the amorphous alkaloid content was that currently available for transformation to the crystallisable alkaloids, while the crystallisable alkaloid content represented the accumulation of the end products of the amorphous alkaloid transformations throughout tree growth. Cambial activity further governed the efficiency or degree of conversion of the amorphous to the crystallisable alkaloids, for in one tree the highest crystallisable to amorphous alkaloid ratios obtained where the tree bent and where the secondary leader arose. In general, the distribution of the alkaloids throughout the bark is governed primarily by the history of the tree as reflected in its form. R. E. S.

Datura Stramonium, Growth Effects produced by 2:4-Dichlorophenoxyacetic Acid applied to the Stems. H. W. Youngken, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 196.) Hydrous wool fat containing 2:4-dichlorophenoxyacetic acid in concentrations varying from 0.0001 to 5 per cent. was applied to the stems of seedlings about 3 in. high and older plants about 8 to 10 in. high. Seedlings treated with 0.0001 and 0.001 per cent. showed no abnormal effects, but higher concentrations produced a systemic effect proportional in degree to the concentration used. The effect was manifested by stunted growth, skin bending and swelling, cellular proliferation and roughening and curling of the leaves. Seedlings did not recover from the effects if the concentration was greater than 1 per cent. Those older plants treated with 1 to 5 per cent. showed, after 45 days, stunted growth, swollen stem bases with warty outgrowths, marked curvature of the stems above the first branching, folding of the leaves, increased pubescence along the leaf veins and petioles, and retardation of flower development, only the lower flowers maturing; other flower buds which formed remained unopened. Two plants treated with 1 to 0.1 per cent. and on which mature fruits formed produced spineless capsules. After three weeks the seeds of these fruits were chocolate brown and either flattened and kidney-shaped, or vermiform to crescent or horn-shaped, and smaller. Older plants treated with 0.1 per cent. or less showed little if any toxic effects. The determination of the total alkaloids of leaves from plants which were 85 days old and had received four

applications of 0.001 per cent. of 2:4-dichlorophenoxyacetic acid showed no significant difference from the control plants. Since, however, stronger concentrations produce somewhat drastic effects on the leaves, the effect of these on alkaloidal formation is being investigated. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Alcohol, Sensitisation to, by Drug. J. Hald and E. Jacobsen. (*Lancet*, 1948, 255, 1001.) Doses of 0.5 to 1.5 g. of diethylthiuramdisulphide [bis(diethylthiocarbanyldisulphide)], $(C_2H_5)_2NC(S).S.S.C(S)N(C_2H_5)_2$ although without effect by themselves produce unpleasant symptoms if alcohol is taken subsequently. The symptoms following 10 to 20 g. of alcohol include flushing of the face, dilatation of the scleral vessels, palpitations and possibly slight dyspnoea. Larger doses of alcohol cause nausea and vomiting. This sensitisation seems to be due to a great increase in blood acetaldehyde which occurs when both the drug and alcohol are taken, but which is absent when either is taken separately. Sensitisation begins about 3 hours after the drug is taken and may last about 48 hours depending on the dose. The drug is stated to be non-toxic by itself and is excreted very slowly. W. W. W.

Alcoholism treated by Sensitising Drug. O. Martensen-Larsen. (*Lancet*, 1948, 255, 1004.) The author has used tetraethylthiuramdisulphide in the treatment of 83 cases of alcoholism. This drug although innocuous by itself causes unpleasant symptoms if alcohol is taken subsequently. 74 of the patients developed distaste for alcoholic drinks as a result of the treatment; 9 refused to continue with it. After physical and psychiatric examination the patient received 1.0 to 1.5 g. of the drug followed by 0.5 g. daily, and informed of the consequences should he drink alcohol, but he is encouraged to try it to show the effect of the treatment. Although heavy drinkers can still take a fair amount of alcohol at the start of the treatment, their tolerance for alcohol soon diminishes and finally all desire for drink seems to be lost. W. W. W.

Analgesics, A Method of Testing in Man. A. J. H. Hewer and C. A. Keele. (*Lancet*, 1948, 255, 683.) Instead of the usual method of testing analgesics by measuring the threshold intensity of some stimulus required to elicit pain, tests have been made on the power to relieve pain experimentally induced by the contraction of ischaemic muscles. By means of a cuff the distal circulation of the arm was cut off and the hand and forearm muscles were then exercised by rhythmically compressing a bulb. After 50 to 60 contractions had been made there was slight or moderate pain in the forearm muscles. The contractions were then stopped and the ischaemia still maintained. Soon the pain began to increase and became intolerable after 8 to 15 minutes. If the cuff was deflated the pain disappeared in a few seconds. Ten grades of pain could be distinguished and are described in units from 1 to 10. The drugs were given when the pain was of the value of 4 to 5 units, gases by inhalation, the others by intravenous injection, thus eliminating differences due to different rates of absorption. It was found preferable, instead of trying to find the smallest dose that would give complete relief from pain of 4 to 5 units of intensity, to find the smallest dose that would produce a definite effect at this stage. For this purpose a narrowing range of doses, well above and below the threshold, were given, until there was only a small differ-

ence between the effective and ineffective doses. Nitrous oxide from 10 per cent. to 40 per cent. in oxygen, and cyclopropane, 2 and 3 per cent. in oxygen, were used. Results are given for the following drugs: (1) morphine (hydrochloride or sulphate); (2) pethidine hydrochloride; (3) *dl*-2-dimethylamino-4:4-diphenyl-heptan-5-one hydrochloride, amidone; (4) *dl*-1-dimethylamino-3:3-diphenyl-hexan-4-one hydrochloride (Hoechst 10582); (5) *dl*-2-dimethylaminomethyl-3:3 diphenyl-hexan-4-one hydrochloride (iso-amidone); (6) *dl*-2:1-morpholino-4:4 diphenyl-heptan-5-one hydrochloride (C.B. 11); (7) procaine hydrochloride; (8) thiopentone sodium; (9) benadryl hydrochloride; (10) *N*-*p*-methoxybenzyl-*N*-dimethylaminoethyl-2-amino-pyridine maleate (neointergan, antistin, pyranisamine maleate). (11) *N*-phenyl-*N*-benzyl-2-methylimidazoizoline (antisan); (12) tetraethylammonium bromide. The results show great variations between the 4 persons studied and clinical differences in response to these drugs can, in part, be ascribed to differences in sensitivity towards them. It was found, for example, that amidone and C.B.11 were both 30 times as potent as pethidine, while in another subject morphine and amidone were both 10 times as potent as pethidine but only 1/10 as powerful as C.B.11. Again, in another subject morphine, amidone and C.B.11 were about equally analgesic and were 20 to 25 times more potent than pethidine

H. F.

Calciferol by Intramuscular Injection. T. Lightbound (*Lancet*, 1948, 255, 1010.) Initially, patients with lupus vulgaris, lupus verrucosus, erythema induratum and normal controls were treated orally with 150,000 I.U. daily of calciferol for 6 months. The dose had to be reduced in some patients owing to nausea and vomiting. To overcome this, intramuscular injections of 600,000 I.U. were given thrice weekly for 3 weeks and then twice weekly. The intramuscular route is preferred because results were more rapid, pigmentation was absent, there were no toxic symptoms, there was little or no hypercalcaemia, less chance of reduced packed-cell volume and of raised blood urea. All cases, however, showed diminished kidney function (urea clearance test), and therefore treatment should not exceed 4 months without a rest period

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Chloroquine, Chronic Oral Toxicity of. O. G. Fitzhugh, A. A. Nelson and O. L. Holland. (*J. Pharmacol.*, 1948, 93, 147.) A 2-year chronic toxicity study with rats fed on diets containing from 100 to 1,000 p.p.m. of chloroquine showed that the toxicity of the drug was very slight or questionable at 100 p.p.m. and became progressively more severe with each increase in dosage. There was a significant retardation of growth at a concentration of 400 p.p.m. A progressive increase in mortality occurred at dosage levels of 200 p.p.m. or more, and 800 and 1,000 p.p.m. caused early death of all animals. The outstanding haematological change was a leucocytosis, predominantly neutrophilic, marked in the group on 800 p.p.m., less striking in the group on 400 p.p.m., and scarcely noticeable in the group on 200 p.p.m. there was increase in the haemoglobin concentration and erythrocyte counts in the rats on 800 p.p.m. Histopathological changes increased from very slight or absent in rats on 100 p.p.m., to marked in those on 800 and 1,000 p.p.m. the two prominent lesions at toxic doses were a slow focal necrosis of striated muscle, especially cardiac, and a moderate degree of centrilobular hepatic necrosis and fibrosis. In relation to bodyweight of the rat, the lowest dosage of chloroquine which produced slight toxic effects in some animals corresponds to approximately 4 mg./kg./day for 2 years. This corresponds

applications of 0.001 per cent. of 2:4-dichlorophenoxyacetic acid showed no significant difference from the control plants. Since, however, stronger concentrations produce somewhat drastic effects on the leaves, the effect of these on alkaloidal formation is being investigated. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Alcohol, Sensitisation to, by Drug. J. Hald and E. Jacobsen. (*Lancet*, 1948, 255, 1001.) Doses of 0.5 to 1.5 g. of diethylthiuramdisulphide [bis(diethylthiocarbonyl)disulphide], $(C_2H_5)_2NC(S).S.S.C(S)N(C_2H_5)_2$, although without effect by themselves produce unpleasant symptoms if alcohol is taken subsequently. The symptoms following 10 to 20 g. of alcohol include flushing of the face, dilatation of the scleral vessels, palpitations and possibly slight dyspnoea. Larger doses of alcohol cause nausea and vomiting. This sensitisation seems to be due to a great increase in blood acetaldehyde which occurs when both the drug and alcohol are taken, but which is absent when either is taken separately. Sensitisation begins about 3 hours after the drug is taken and may last about 48 hours depending on the dose. The drug is stated to be non-toxic by itself and is excreted very slowly. W. W. W.

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approximately to the prophylactic dosage in man; however, taking the length of time into consideration, the amount of chloroquine that will produce toxic effects in rats is above the therapeutic or prophylactic dose for man. The toxicity of chloroquine was found to be slightly less than that of mepacrine; at the low dosage level of 4 mg./kg./day there was no noticeable difference between the toxicities of the two substances. S. L. W.

Dimercaprol in the Treatment of Experimental Lead Poisoning in Rabbits. F. G. Germuth and H. Eagle. (*J. Pharmacol.* 1948, 92, 397.) Rabbits which had received 5 consecutive daily subcutaneous injections of lead acetate in a dosage of 240 mg./kg. all died in from 3 to 40 days, the survival time averaging 26 days after the last injection. A series of animals receiving this dosage of lead acetate were then treated for 5 days with dosages of dimercaprol varying from 20 to 80 mg./kg. daily. Dimercaprol significantly hastened their death, the average survival time at the smallest dosage of dimercaprol being reduced from 26 days to 15 days, and at the highest dosage from 26 days to from 1 to 12 days, the mortality being greatest within the first four days. This experiment was carried out in the hottest part of the summer; in a second similar experiment carried out in the winter months dimercaprol did not accelerate death but it still failed to exert a protective action. Similar results were found in acute lead poisoning induced by intravenous injections of lead acetate, the animals treated with dimercaprol dying approximately in the same time as the untreated controls. Further experiments to determine the effect of dimercaprol on urinary lead excretion showed that it caused a striking increase. For two hours after a single injection of dimercaprol at 20 mg./kg. the urinary excretion of lead increased 11- to 40-fold in animals with a subcutaneous depot and 3- to 7-fold in animals injected intravenously, this favourable effect lasting for 4 hours after a single injection. The magnitude of the lead-excretion response decreased with each additional injection of dimercaprol, suggesting that only a small proportion of the lead injected could be dissociated by dimercaprol from its combination with the tissues. The reason for the failure of dimercaprol to protect the animals in spite of increased urinary lead excretion may be due in part to the fact that only a small portion of the total body store of lead is mobilised and the amount eliminated is too small to effect the outcome. A second reason for failure may be the fact that the lead mobilised by dimercaprol is shown to form a lead-dimercaprol complex which is almost as toxic as lead acetate itself, which may explain why in some experiments the administration of dimercaprol actually accelerated death, since the lead complex when formed may act on other organs more vulnerable to the toxic effects of lead, or more vital to the host, than were the tissues in which the lead was originally deposited. In spite of these results, the authors consider, in view of the striking effect of dimercaprol on the urinary excretion of lead, its cautious therapeutic trial in man is justified. S. L. W.

Hetrazan, Mode of Action in Filariasis. F. Hawking, P. Sewell and P. Thurston. (*Lancet*, 1948, 255, 730.) Experiments were carried out on cotton-rats infected with *Litomosoides carinii*, to study the mode of action of a new compound, hetrazan (1-diethylcarbamy-4-methylpiperazine), which has been introduced for the treatment of human filariasis due to *Wüthchereria bancrofti*. Intravenous injection of 6 mg./100 g. was followed by rapid diminution of the microfilariae, 80 per cent. disappearing in 1 minute and over 90 per cent. in 2 minutes. Microfilariae surrounding

the adult worms in the pleural cavity remain active despite intensive treatment and form a reservoir from which the supply in the blood is constantly replenished. Hetrazan has little effect on the adult worms. Experiments *in vitro*, and the histological examination of the distribution of the microfilariae in the different organs suggests that hetrazan has an opsonin-like action on the microfilariae and renders them susceptible to destruction by the reticuloendothelial system. Microfilariae *in vitro*, and in the pleural cavity are not in contact with phagocytes and are not so rapidly affected by hetrazan. E. N. I.

Niaara: a Digitalis-like Colombian Arrow-Poison. K. Mezey, C. Uribe-Piedrahita, J. Pataki and J. Huertas-Lozano. (*J. Pharmacol.*, 1948, 93, 223.) Niaara, an arrow-poison from Colombia, is the latex of the tree "Paeuru-niaara," or "poison tree," *Ogcodeia ternstroemiiflora* Midbr. A white, amorphous principle, niaarin, having chemical and pharmacological properties characteristic of the cardiac glycosides, has been isolated from this latex. The intravenous LD50 in cats is 0.21 mg./kg.; it is poorly absorbed from the gastro-intestinal tract. Seven cases of congestive heart failure in man were successfully treated with niaarin, injected intravenously daily in doses of 0.50 to 0.75 mg. for the first two days, and then 0.25 mg. daily for a further 2 or 3 days. The therapeutic effect was evident within less than 24 hours after administration. On the whole, niaarin compares closely with strophanthin in rapidity and brevity of action. Cumulation is not important when 0.25 mg. is given as the daily maintenance dose. No side effects were noted in these cases, and no curare-like action is produced. Although niaarin is an effective therapeutic agent, the necessity for intravenous administration limits its field of usefulness. On the other hand, the actions of niaarin develop with remarkable rapidity which may occasionally be desired. S. L. W.

Ouabagenin. K. K. Chen, R. C. Anderson and H. M. Worth. (*J. Pharmacol.*, 1948, 93, 156.) Employing a crystalline form of ouabagenin, the authors determined it to be approximately one-half as active on the heart as ouabain in cats and about one-third as active in frogs. It is more effective than ouabain in causing vomiting of non-anæsthetised cats, weight for weight, and the emetic dose is therefore no measure of the cardiac activity when different compounds are compared. Unlike digitoxigenin, it does not cause convulsions in cats or frogs. S. L. W.

Penicillin Treatment of Nasopharyngeal Diphtheria. E. W. Bixby. (*Amer. J. med. Sci.*, 1948, 215, 509.) A series of 139 cases of diphtheria in young men, all of whom had been previously immunised according to the U.S. Army Schedule, were selected for study. Although the typical grey membrane was seldom found, each case included in the report had a positive nasal and/or pharyngeal culture for *Corynebacterium diphtheria*, and gave a confirmed positive virulence study. Every case received 100,000 units of diphtheria antitoxin intramuscularly, and general routine treatment. Of the 88 cases receiving only this treatment 53 (60 per cent.) developed a carrier state after two weeks. To the remaining 51 cases, penicillin was administered intramuscularly immediately on confirmation of the presence of *C. diphtheria*, in doses of 20,000 units every 3 hours for 50 doses. Of these, 43 (79 per cent.) had permanently negative cultures after 2 weeks. The average time required in hospital by patients with a 2-week negative culture was 35 days compared with an average of 50 days for those cases with a two-week

positive culture. In contrast to these results, the use of penicillin later in 40 cases of the two-week positive group who had previously received only the routine treatment was not successful, 60 per cent. remaining positive. The incidence of post-diphtheritic complications was not affected by penicillin although the severity of complications was modified. H. T. B.

Sodium Salicylate, Cutaneous Absorption of. G. Valette and R. Césari. (*Ann. pharm. Franc.*, 1948, 6, 16.) The object of this investigation was to apply the characteristic elimination of sodium salicylate in the urine, to the study of the factors governing the absorption by the skin of non-liposoluble substances. The addition of eucalyptol markedly enhances the cutaneous penetration of sodium salicylate; the degree of penetration is dependent on the proportion of eucalyptol, but when the proportion exceeds 20 per cent. of the volume of the solution it produces an irritant effect. Sodium salicylate is hydrolysed in solution; when eucalyptol is added to such a solution it dissolves the salicylic acid liberated, with which it gradually becomes charged and penetrates the skin, and the irritation observed is thus attributable not to the eucalyptol but to the salicylic acid dissolved in it. This action of eucalyptol is less marked in alcoholic than in aqueous solutions. The addition of an emulsifying agent to a mixture of eucalyptol and solution of sodium salicylate does not increase the effect produced, though the results are better with oil-in-water than with water-in-oil emulsions. The renal excretion of sodium salicylate after cutaneous application of an aqueous solution containing eucalyptol, follows almost the same rhythm as after administration of the salt orally or subcutaneously, elimination reaching its maximum in 3 hours and continuing for about 48 hours. The alkalisation of solutions of sodium salicylate (to pH 8.4) was found to hinder the cutaneous penetration of the salt, while acidification (to pH 4.6) was found to increase it. S. L. W.

Sulphetrone, Pharmacology and Chemotherapy of. G. Brownlee, A. F. Green and M. Woodbine. (*Brit. J. Pharmacol.*, 1948, 3, 15.) Sulphetrone is 4 : 4'-bis(γ -phenyl- n -propyl-amino)diphenylsulphone-tetra-sodium sulphonate, an amorphous material containing, when air-dried, from 5 to 7 per cent. of water. It is insoluble in alcohol and other organic solvents, but is exceedingly soluble in cold water to give a syrup; 20 and 40 per cent. w/v solutions are stable when neutral or slightly alkaline, and may be autoclaved. A 10 per cent. w/v solution is isotonic with 0.91 per cent. sodium chloride solution, and hypertonic solutions up to 60 per cent. are readily obtained. In mice and dogs sulphetrone has an acute toxicity many times less than that of sulphanilamide. Very large doses can be given by mouth to mice and dogs without producing symptoms or pathological changes, but similar doses in rabbits produce anaemia. It causes hyperaemia and hyperplasia of the thyroid gland. Sulphetrone is not hydrolysed to diaminodiphenylsulphone in the body. When given orally or parenterally, it raises the alkali reserve of the plasma in the rabbit and the dog, but over a period of time, equilibrium is established. When given by mouth not only is sulphetrone the least toxic of the sulphones, but is also less toxic than any of the sulphonamides. It is only slowly absorbed from the intestinal tract; when given intravenously it is excreted in the urine almost completely in 24 hours, but when given orally only 75 per cent. is excreted in the same time. The drug is conjugated in the experimental animal or in man. It penetrates all tissues with extreme rapidity, with the exception of the brain, and is present in them in about the same concentration as in blood, but it enters the cerebro-spinal fluid rather more slowly than do other sulphonamides. It has no action

on smooth muscle, heart, blood pressure or respiration. Antibacterial *in vitro* studies show sulphetrone to approach the efficiency of diaminodiphenylsulphone against avian, bovine and human strains of *Mycobacterium tuberculosis*; blood from guinea-pigs which had previously received parenteral sulphetrone inhibits *in vitro* strains of virulent mycobacteria. The authors conclude that sulphetrone may prove effective in the treatment of experimental tuberculosis in laboratory animals, and that its administration to man in large doses for protracted periods is a practical possibility. S. L. W.

Sulphetrone, Treatment of Tuberculosis with. D. G. Madigan. (*Lancet*, 1948, 255, 174.) In 70 cases of tuberculosis affecting different organs, sulphetrone was given for periods varying from a few days in tuberculous meningitis to eighteen months in more chronic cases. A blood-sulphetrone level of 7.5 to 10 mg./100 ml. should be aimed at by a scheme of gradual dosage. A suitable initial dose for adults is 1.5 g. daily (0.5 g. eight-hourly) for the first week, and 3 g. daily (0.5 g. four-hourly) for the second, the daily dose then being increased by 1 to 2 g. each week until the required blood level is reached, usually with 6 to 10 g. daily. If given intramuscularly combined with streptomycin, for miliary tuberculosis or tuberculous meningitis, 0.05 g./kg. of bodyweight every 4 to 6 hours for the first 24 hours is suitable, increased to 0.1 g./kg. during the second 24 hours; when the meninges are involved, levels above 5 mg./100 ml. may cause vomiting. It is essential to give iron and brewers' yeast for a fortnight before and throughout sulphetrone treatment to avoid hypochromic and nutritional anaemia: the yeast also prevents the onset of peripheral neuritis, occasionally seen. Even so, a residual hæmolytic anaemia develops and continues throughout treatment leading to a fall in hæmoglobin content to a level as low as 60 per cent.; should it fall below this figure the sulphetrone should be withdrawn. Besides a weekly check of red cells and hæmoglobin, there should be a weekly estimate of blood-sulphetrone, which should not exceed 12.5 mg./100 ml. Danger signals are continuous headache, loss of appetite, nausea and vomiting, gastro-intestinal discomfort, dizziness and mental confusion; these conditions are associated with high blood-sulphetrone levels and measures should be taken to hasten elimination of sulphetrone by giving fluids by all routes. Stasis also should always be guarded against. In general, no beneficial effect was detected from sulphetrone therapy of acute infections, but improvement was observed in chronic lesions. Thus, 12 out of 17 cases of acute pulmonary fibrocaceous disease, and 13 out of 22 chronic cases improved. All of 4 cases of primary pulmonary tuberculosis, and 6 out of 8 strictly exudative lesions, improved. All of 4 in the chronic hæmatogenous group and 3 out of 4 in the productive pulmonary infiltrative group improved. In general, all exudative phases of infiltrative disease were halted and reversed by sulphetrone. The need for long-continued courses is emphasised and routine laboratory control is essential. Sulphetrone is useful as an adjuvant with definite objectives in view. S. L. W.

Thenylene: A New Antihistamine Compound. A. S. Friedlaender and S. Friedlaender. (*Amer. J. med. Sci.*, 1948, 215, 531.) A new antihistamine compound, N-(α -pyridyl)-N-(α -thenyl)-N', N'-dimethylethylene-diamine hydrochloride, has been synthesised. Under the name of thenylene (of histadyl) it has been examined to ascertain its effectiveness in preventing fatal histamine shock and anaphylaxis in guinea-pigs, and in alleviating allergic symptoms in man. A protective dose of 3 mg./kg. was administered intraperitoneally to male guinea-pigs 15 minutes prior to the intravenous injection of histamine. This dose protected all animals against one lethal

dose of histamine, while 50 per cent. survived approximately 8 lethal doses. Marked protection was given against fatal anaphylaxis in guinea-pigs sensitised by the intraperitoneal injection of 0.1 ml. of normal horse serum, followed 12 days later by a shock dose of 1 ml. of the same serum intravenously. Of the control animals 100 per cent. died, while only 20 per cent. of fatalities occurred amongst a group receiving 1 mg./kg. 15 minutes before the dose of antigen. The clinical action was studied in 117 patients with one or more allergic complaints, in doses of 100 mg. for adults 4 times daily, or as necessary when symptoms were intermittent. Children were given one-quarter to one-half this dosage. Symptomatic relief was obtained in many cases of urticaria, hay fever and perennial allergic rhinitis. Results in asthma were not striking. Mild side effects occurred in 25 per cent. of patients, but rarely affected administration of the drug. Drowsiness was most common, occurring in 13 patients, and vertigo, headache, gastro-intestinal distress and dryness of mucous membranes were also reported. Toxic symptoms were usually relieved by a reduction of dosage.

H. T. B.

BACTERIOLOGY AND CLINICAL TESTS

Antibiotics, Induced Resistance of *Staphylococcus aureus* to. J. W. Klimek, C. J. Cavallito and J. H. Bailey. (*J. Bact.*, 1948, 55, 139.) It is known that many antibacterial substances are inactivated by various thiol compounds, some reacting rapidly with a large number of -SH compounds, some with cysteine or related β -aminoalkane thiols only, and others displaying reactions intermediate between these two. A study of the development of resistance of *Staphylococcus aureus* to several antibiotics was undertaken to determine whether a correlation existed between development of resistance and the known reactivity of the antibiotics with thiol compounds. The antibacterial agents studied were penicillin, streptomycin, pyocyanin, gliotoxin, aspergillid acid, mercuric chloride, and the active principles of *Allium sativum*, *Asarum canadense* and *Arctium minus*. The susceptibility of *S. aureus* to the antibiotics was determined by growing the organism in a series of beef broth cultures containing increasing quantities of the test agents. Results of the experiments are demonstrated in figures, and show the ability of *S. aureus* to develop rapid and marked resistance to penicillin, streptomycin and the active principle of *Asarum canadense*. The organism developed an intermediate degree of resistance to pyocyanin and gliotoxin, very little resistance to mercuric chloride or the active principle of *Arctium minus* and no resistance to aspergillid acid. These results run parallel with the degree of specificity of reactivity with sulphydryl groups. The more selective the antibiotic as to the type of -SH compound with which it will react, the more readily does it induce bacterial resistance. Reversibility of resistance occurs with antibiotics which react non-selectively with thiols, while the resistance induced by antibiotics reacting selectively with thiols is likely to be non-reversible.

H. T. B.

Streptomycin. Activity in Presence of Serum and Blood. E. B. Schoenbach and C. A. Chandler. (*Proc. Soc. exp. Biol. N.Y.*, 1947, 66, 493.) Bactericidal tests were carried out on the growth of *Staphylococcus aureus* in the presence of streptomycin, various factors possibly affecting such tests, i.e. phagocytosis, hæmolysis, immune serum and labile

[Continued on page 265]

PHARMACOPŒIAS AND FORMULARIES

SCANDINAVIAN PHARMACOPŒIA COMMISSION

At a Conference held, on the invitation of the Swedish Government, at Stockholm in November 1948, a joint Pharmacopœia Commission for the Scandinavian countries was formed. This consists of 3 representatives of each of the Pharmacopœia Commissions of Denmark, Norway and Sweden, nominated by the Governments of these countries. It is hoped that Finland will also join. The chairman is Prof. G. Ahlgren, Sweden, and the General Secretary, Dr. F. Reimers, Denmark.

Formation of the joint commission has given a permanent organisation to the co-operation between the Pharmacopœia Commissions of the Scandinavian countries which has been kept up by occasional conferences throughout a number of years. While, in the earlier period of co-operation, it was only possible to obtain agreement on single points, work will now be directed towards gradually obtaining such complete agreement that a joint Scandinavian Pharmacopœia can be published.

That would have many advantages. The medical and pharmaceutical professions are so close to one another in these countries, and the difference in language is so small, that any difference between the Pharmacopœias should be avoided, as this may prove a hindrance in connection with scientific literature, text-books, and education, and also may give rise to difficulties in understanding prescriptions written by doctors of the other countries. Further, all the countries manufacture chemicals to a limited extent only and most of the requirements are imported from the same suppliers abroad. It will also be a great advantage that work can be shared among the Pharmacopœical laboratories of these countries, for initial attack on problems and for checking of results.

Instructions to the joint commission have been drafted and are under consideration by the respective Governments. One important decision is that the names of drugs to be used in the National Pharmacopœias shall be decided by the joint Commission.

F. REIMERS.

ABSTRACTS (continued from page 264)

constituents being also investigated. A susceptible strain of the organism was used and also a variant of that strain resistant to 1,000 $\mu\text{g.}$ of streptomycin. In broth, the bacteriostatic range of streptomycin for the susceptible strain was not affected by the presence of the 60 per cent. of human or rabbit serum. When the resistant strain was tested in the presence of 60 per cent. of rabbit serum, as little as 125 $\mu\text{g.}$ 'ml. of streptomycin gave retarded growth with small inocula at 24 hours but not at 48 hours. This transient inhibition was not due to any constituent of the serum, neither was it due to stimulation of phagocytosis with subsequent death of the leucocytes. Possibly the inhibition is due to modification of the nutritional requirements with the acquisition of streptomycin resistance. It is suggested that there are two alternative growth mechanisms available to the organism, one being blocked by streptomycin, the other being insufficiently developed at first but increasing later.

H. T. B.

BOOK REVIEWS

THE STUFF WE'RE MADE OF, by W. O. Kermack and P. Eggleton. (Pp. 356, 8 Plates, 75 Figs. in the Text.) E. Arnold and Co., London, 1948, 10s. 6d.

The second edition of this book is not greatly different from the first, since research during the last eight years has not seriously modified the facts and theories generally accepted before the first edition was published. Additional information has accumulated, but mostly on detail outside the scope of this book. Knowledge of the vitamins has, however, grown so much that the one chapter devoted to them in the first edition has been replaced by two, one being devoted to the B vitamins. The parts played by riboflavine, nicotinic acid, pyridoxine, biotin, etc., in the nutrition of animals have been worked out, and more experiments have been performed on human beings who have volunteered for various dietary treatments. The production of certain factors by the bacteria present in the alimentary tract has been demonstrated and their worth to the animal noted. The destruction or inhibition of these valuable bacteria by drugs given for other purposes has also been shown. The other fresh chapter in this edition deals with the subject of muscle contraction. The action of adenine triphosphate on myosin and *vice versa* forms the basis for a theory of muscle contraction which the writers "make to work" by means of a working model built up of springs and "Lazy-tongs." They end this chapter with the sentence "Nature's engines are indeed miracles of design and construction; their existence would be incredible if they were not so utterly commonplace." The book is full of sound information, reasoned speculation and a way of "looking at the wood and not only at the trees." It is, in fact, so good that the present reviewer's only regret is that in so many places, the writers have tried to make the book "popular" by using frivolous and skittish expressions: e.g., the term "vitamin racket" has been applied to the whole study of vitamins. If it had been applied to the attempt to get anybody and everybody to dose himself liberally with synthetic vitamins, at a high cost in shillings or dollars, no one could have objected to the term. However, the fact that a second edition has been called for is evidence that the style of the writing has been acceptable to the general public for whom it was intended, and who would possibly not have read the book if it had been consistently serious all through.

K. H. COWARD.

THE PRESENTATION OF TECHNICAL INFORMATION, by R. O. Kapp. Pp. 140 and Index, Constable and Co., London, 1948, 6s.

The many problems arising in the writing of good technical English are carefully analysed in this book and much sound advice is offered to writers of papers, reports and reviews. Although the author himself is concerned with the engineering field his remarks apply equally well to other branches of science. The book is based on a series of lectures given at University College, London, a year or so ago. Functional English, which is not to be confused with Basic English, is described and recommended as the language for scientific writings. Its essential purpose is the conveyance of new information, which may be factual, or argumentative, and infer or suggest new lines of thought. The author discusses the

BOOK REVIEWS

problem not only from the writer's viewpoint but also from that of the reader. Thus, in addition to being good English, Functional English must be easily readable. A good introduction with a statement of the terms of reference, a logical arrangement of thought and argument, the selection of material for its relevance to the problem in hand and the presentation of facts at a pace at which they can be understood and remembered are just as important as well-constructed sentences. A well-written report should not only give information, but should also stimulate thought in the reader. There are useful chapters on the avoidance of circumlocutions and the use of generalisations, qualifications and metaphor. In writing this book the author has put his own principles into practice and the result is a well-written, interesting and stimulating publication which should be read by both students and post-graduate workers. The adoption by students of Professor Kapp's suggestions at an early stage would go far towards the production of laboratory notes of the high standard spoken of so frequently by examiners

BOOKS RECEIVED

THE CHEMICAL FORMULARY by H. Bennett, Vol. VIII. Pp. 428 and Index, Chapman and Hall, Ltd., London, 1948, 42s.

PRINCIPLES OF BIOLOGICAL ASSAY by C. W. Emmens. Pp. 204. Chapman and Hall, Ltd., London, 1948, 21s.

THE BACKGROUND OF THERAPEUTICS by J. H. Burn. Pp. 335 and Index, Oxford Medical Publications, London, 1948, 22s. 6d.

GRUNDLAGEN DER PHARMAKOLOGIE by K. W. Merz. Pp. 274 and Index, Wissenschaftliche Verlagsgesellschaft M.B.H., Stuttgart, 1948. 4th Ed.

PRECIS DE CHIMIE TOXICOLOGIQUE by F. Schoofs. Pp. 513 and Index, Les Presses Universitaires de Liège, 1948, 2nd ed.

LA CHIMIE DES VITAMINES ET DES HORMONES by J. Sivadjin, Vol. I. Pp. 479. Gauthier-Villars, Paris. 1948, 3rd ed.

PRACTICAL PHARMACOGNOSY by B. E. Hébert and K. W. Ellery. Pp. 365 and Index, Ballière, Tindall and Cox, London, 1948. 21s.

TRACE ELEMENTS IN FOOD by G. W. Monier Williams. Pp. 498 and Index, Chapman and Hall, Ltd., London, 1949, 30s.

LETTERS TO THE EDITOR

The Specific Rotation of Emetine Hydrochloride

SIR,—The following observations, which seem of sufficient interest to record, were made when we were asked to examine a sample of emetine hydrochloride according to the standard of the French Codex (6th edition, 1937).

Emetine hydrochloride of the French Codex is required to have a specific rotation of $+53^\circ$ when determined on a 2 per cent. solution of the anhydrous salt in chloroform but, under these conditions and using chloroform B.P. as solvent, our specimen had $[\alpha]_D^{20^\circ} +47.5^\circ$. It is noteworthy that Carr and Pyman¹ give $+53^\circ$ as the specific rotation for the anhydrous salt in chloroform.

As is well known, pure chloroform is unstable and for this reason it is customary to add a small percentage of alcohol as preservative; thus chloroform of the B.P. is required to contain 1 to 2 per cent. v/v. of alcohol. It occurred to us that the alcohol content of the chloroform used as solvent might influence the specific rotation of emetine hydrochloride. Accordingly, we prepared some pure chloroform, free from alcohol, and determined the specific rotations of three batches of emetine hydrochloride, dried *in vacuo* over phosphorus pentoxide for 48 hours, in this solvent. The results for $[\alpha]_D^{20^\circ}$ for 2 per cent. solutions of these samples in pure chloroform were: (1) $+59.64^\circ$, (2) 59.98° and (3) 59.98° . A sample of chloroform B.P. was then prepared by adding 1.5 per cent. v/v. of absolute alcohol to our pure chloroform and the specific rotation of anhydrous emetine hydrochloride (sample 1) determined in this solvent. The figure obtained was $+47.3^\circ$ (c. 2.0).

It is evident that the actual figure for the specific rotation is greatly influenced by the alcohol content of the chloroform used as solvent and, on this account, we believe that it is more reliable to carry out the determination with an aqueous solution. In our experience, emetine hydrochloride has $[\alpha]_D^{20^\circ} +17.8^\circ$ when determined on an accurately prepared 5 per cent. w/v. solution of the anhydrous salt in water. Indeed, we have examined hundreds of samples in aqueous solution and the specific rotations have never deviated from $+17.8^\circ$ by more than a few tenths of a degree. Further evidence of the erratic results, which may be obtained when using chloroform as solvent, was afforded when we decided to repeat our work a week after the original experiments. Our pure chloroform had then become slightly acid in reaction owing to decomposition. The specific rotation of anhydrous emetine hydrochloride in this solvent was $+72.86^\circ$, but after purification of the chloroform a figure of $+59.81^\circ$ was obtained.

The Wellcome Chemical Works, Dartford.
January 27, 1949.

A. E. BEESLEY.
G. E. FOSTER.

REFERENCE

1. Carr and Pyman, *J. chem. Soc.*, 1914, 105, 1604.

NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Abidex Drops* are a stable, non-oily and non-alcoholic multivitamin solution intended for the prevention and treatment of vitamin deficiencies, particularly in children. Each 10 min. (30 drops) contains: vitamin A 5000 I.U., vitamin D 1000 I.U., aneurine hydrochloride 1 mg., riboflavine 0.4 mg., nicotinamide 5 mg., ascorbic acid 25 mg. The drops are well tolerated by infants and children, and may be taken directly or mixed with milk, fruit juice, soup or other foods; they may also be safely added to the contents of the infant's feeding-bottle. The average daily dose for infants under 1 year is 5 min., and for older children 10 min. The drops are supplied in 10-ml. bottles, with a dropper. S. L. W.

Bplex Elixir* is an aqueous extract of rice bran with members of the vitamin B complex added so that each ml. contains: aneurine hydrochloride 0.125 mg., riboflavine 0.25 mg., nicotinic acid 1.25 mg., calcium pantothenate 0.625 mg., together with significant amounts of choline, inositol and other factors; it also contains 16 per cent. of alcohol. The dose is 2 fl. dr. daily, or as prescribed. Bplex capsules contain: aneurine hydrochloride 1 mg., riboflavine 0.80 mg., nicotinamide 10 mg., pyridoxine 0.012 mg., and pantothenic acid 0.013 mg. The dose is 3 or more capsules daily. The elixir is issued in 4-ounce bottles and the capsules in bottles of 50. S. L. W.

Bismuth Sodium Triglycollamate. (*New and Non-official Remedies, J. Amer. Med. Ass.*, 1948, 138, 749.) Bismuth sodium triglycollamate ($C_{24}H_{28}O_{25}N_4BiNa_7$) is a double salt of bismuthyl sodium triglycollamate and disodium triglycollamate, containing about 18.3 per cent. of Bi. It is a white, odourless, stable, crystalline powder with a saline taste, soluble in water and insoluble in organic solvents. It must comply with limit tests for carbonate, chloride, sulphate, nitrate, lead, copper, silver, arsenic and moisture. It is assayed by igniting at $700^{\circ}C$., precipitating the bismuth with hydrogen sulphide and weighing the bismuth sulphide obtained. Bismuth sodium triglycollamate is used for the oral administration of bismuth in the treatment of syphilis, alone, or in conjunction with other antisypilitics. It has the same contraindications as other bismuth preparations. The total daily dose is 0.82 g. (equivalent to about 150 mg. of bismuth) to 1.23 g. (225 mg. of bismuth) given in 2 or 3 divided doses. It is supplied in the U.S.A. as tablets containing 0.41 g. (75 mg. of bismuth) under the trade-name Bistriplate. G. R. K.

Dermogestic Ointment* contains, in each 100 g., calamine 8 g., benzocaine 3 g., and hexylated metacresol 0.05 g., in a vanishing cream base. It is a bland, non-greasy, analgesic ointment for the relief of irritant skin conditions. It is supplied in 1 oz. collapsible tubes. S. L. W.

Dihydrocodeinone Bitartrate. (*New and Non-official Remedies, J. Amer. med. Ass.*, 1948, 138, 820.) Dihydrocodeinone bitartrate ($C_{18}H_{21}O_3N$, $C_4H_6O_6 \cdot 2H_2O$) is the hydrated bitartrate of dihydrocodeinone, and occurs as a white, odourless, crystalline powder, soluble in water and slightly soluble in alcohol; a 5 per cent. aqueous solution has pH about 3.5. It is identified by the m. pt. of the base and the oxime, and is distinguished from morphine

by treatment with a solution of selenious acid in sulphuric acid: dihydrocodeinone gives a green colour, which changes to blue and then slowly to purple, whereas morphine gives a blue colour, which changes to green and then to brown. Dihydrocodeinone bitartrate in sulphuric acid solution gives no colour with ferric chloride (distinction from codeine). It is assayed by dissolving the precipitated base in excess of sulphuric acid and titrating back with sodium hydroxide. It has an action similar to that of codeine, but weight for weight is more active and more liable to cause addiction. It is used to allay cough in the same manner as codeine, but it has no clear-cut advantage over the latter. The adult dose is 5 to 15 mg. given 3 or 4 times in 24 hours; children of 2 years and over may be given half the adult dose and younger children one quarter the adult dose. It is supplied in the U.S.A. under the trade-name "Hycodan."

G. R. K.

Isobornyl Thiocynoacetate, -Technical. (*New and Non-official Remedies, J. Amer. med. Ass.*, 1948, 136, 1099.) Isobornyl thiocynoacetate contains 82 per cent. or more of $C_{13}H_{19}ON_2S$, mol. wt. 253.35, with other terpenes. It is a yellow, oily liquid; odour terpene-like; very soluble in alcohol, benzene, chloroform and in ether, practically insoluble in water. Refractive index, 1.512; weight per ml. at 20°C., 1.1465 g.; acid number, 1.19. When 5 ml. of 2N alcoholic potassium hydroxide is added to 25 mg. of isobornyl thiocynoacetate-technical and the solution heated for 5 minutes acidified with diluted sulphuric acid, and a few drops of ferric ammonium sulphate test solution added, a red colour develops. When 1 ml. of a 10 per cent. w/v test solution of ferrous sulphate is added to the heated mixture of isobornyl thiocynoacetate and alcoholic potassium hydroxide and the solution is warmed for another 5 minutes and acidified with diluted sulphuric acid, a blue colour develops. On adding 1 ml. of 2N alcoholic potassium hydroxide to a 5 ml. of a 10 per cent. alcoholic solution, a yellow colour, which rapidly changes to deep orange, is formed. Isoborneol crystals, m.pt. 200° to 205°C., are obtained by hydrolysis with potassium hydroxide. Nitrogen, by the Kjeldahl method, should not be less than 4.6 per cent., which is equivalent to an isobornyl thiocynoacetate content of 80 per cent. An oily emulsion containing 5 per cent. isobornyl thiocynoacetate-technical and 0.6 per cent. of dioctyl sodium sulphosuccinate, is an effective pediculicide.

L. H. P.

Myanesin* is a proprietary brand of α : β -dihydroxy- γ -(2-methylphenoxy)propane, and is the most effective member of a series of compounds possessing muscle-relaxing properties. It acts centrally by diminishing the reflex excitability of the spinal cord, and not on the myoneural junction as does curare. It does not act on the higher centres, and consciousness is not affected. Even in paralysing doses it does not produce respiratory arrest. Its use is indicated whenever complete relaxation of the abdominal musculature is required surgically without resorting to a deep plane of anaesthesia. It is suitable for use with any general anaesthetic or combination of anaesthetics, and is administered intravenously, usually in doses of 5 to 10 ml. of a 10 per cent. solution; the injection is given slowly 1 or 2 minutes before relaxation is required, and the effect of the dose lasts for 20 to 30 minutes. In patients with impaired renal function, or where a prolonged operation is being carried out, it is best administered as a 2 per cent. solution (prepared by the addition of the contents of a 10 ml. ampoule to 40 ml. of normal saline). Myanesin is issued in boxes containing 3 or 12 ampoules each containing 10 ml. of 10 per cent. solution.

S. L. W.

NEW REMEDIES

Panlittol* tablets contain $2\frac{1}{2}$ grains of pancreatic extract and 1/10 grain of thyroid (B.P. 1932). The tablets are recommended for the control of essential hypertension, and in the treatment of disorders such as Raynaud's syndrome in which there is peripheral vascular spasm. The recommended initial dosage is 1 tablet 3 times daily, taken half an hour before meals; if necessary, the dose may be increased to 2 or 3 tablets 3 times daily. The administration of the tablets should be combined with the routine measures for the care of the hypertensive patient. Panlittol tablets are issued in bottles of 24, 100, 500 and 1,000. S. L. W.

Phytdermine* cream and powder are preparations for the treatment and prevention of fungous infections of the skin, particularly athlete's foot. The cream, which is applied to the affected part at night after bathing, contains phenylmercuric acetate 0.167 per cent., terpineol 1 per cent. and salicylic acid 3 per cent. in a water-miscible base. The powder, which is dusted into socks and shoes in the morning, contains methyl *parahydroxy*-benzoate 5 per cent., salicylic acid 5 per cent. and perfumed talc 90 per cent. S. L. W.

Promanide* is *pp'*-diaminodiphenylsulphone-*N,N'*-di-dextrose sodium sulphonate (promin) in the form of a jelly containing 5 per cent. in a water-soluble tragacanth base, for topical application in the treatment of accessible tuberculous lesions, or of a 5 per cent. water-soluble ointment for surface application in conditions such as the ulcerative type of lupus. From 3 to 10 ml. of the jelly or ointment may be applied 2 or 3 times a week or more often, or the jelly may be injected into the abscess or sinus. Promanide jelly and ointment are supplied in 2-oz. jars. S. L. W.

Promin* is *pp'*-diaminodiphenylsulphone-*N,N'*-di-dextrose sodium sulphonate and is employed by intravenous injection in the treatment of leprosy. The treatment must be continued over a period of many months, the average intravenous dose being from 2 to 5 g. (from 5 to 12.5 ml. of promin solution), administered daily for 6 consecutive days and omitted on the seventh, with an interval of 1 week at the conclusion of each 2 weeks' treatment. Serious toxic reactions are rare, but patients should be under constant observation and blood counts taken every 2 weeks. Encouraging results are also claimed for the use of promin in tuberculosis. It is not a sterilising drug in tuberculosis, and the terminal stages of the disease are not improved, but arrest or temporary stabilisation of the disease can be expected in selected cases of early non-destructive lesions. The best results have been obtained in the preparation of patients for surgical procedure. Promin is supplied in sterile aqueous solution in 5 ml. ampoules containing 2 g. and in 12.5 ml. ampoules containing 5 g. It is issued in boxes of 25 ampoules. S. L. W.

***d*-Tubocurarine Chloride.** (*New and Non-official Remedies, J. Amer. med. Ass., 1948, 138, 821.*) *d*-Tubocurarine chloride ($C_{36}H_{44}O_6N_2C_{12}, 5H_2O$) is the crystalline chloride of a quaternary base alkaloid obtainable from the bark and stems of *Chondodendron tomentosum* and related species (a tentative structural formula is given). It is a colourless or yellowish-white to grey or light brown, odourless, crystalline powder, soluble in water, slightly soluble in alcohol, and almost insoluble in chloroform and ether; m. pt. about 265° to $278^{\circ}C$. A dilute solution gives a brilliant blue colour when treated with Folin-Ciocalteu phenol reagent and sodium carbonate and heated in a water-bath. Other identification tests depend upon the production of a pink

(Continued on page 272)

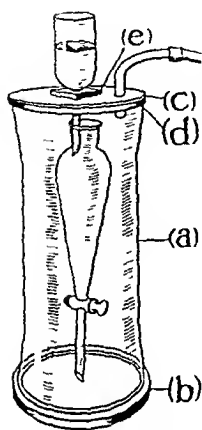
NEW APPARATUS

APPARATUS FOR FILTRATION UNDER REDUCED PRESSURE

By E. D. BANKS

From the Analytical Control Laboratories, May & Baker, Ltd.

Received February 10, 1949



DURING analytical operations, in order to avoid quantitative transference of a filtrate and the further washing involved, it is frequently advantageous to filter under reduced pressure into a vessel not made to withstand it. The apparatus described, which can be used for direct filtration into various sizes of beakers, separators, basins, flasks, etc., is simple and readily constructed. The metal parts are easily made in the laboratory workshop, while the glass tubing and glass discs are standard items which can be obtained from suppliers of chemical glassware. The apparatus consists of a standard 12-in. length of 4-in. diameter butt-ended Pyrex tubing (a) of approximately $\frac{1}{8}$ -in. wall sealed at the bottom with a 6-in. glass inspection-disc (b) cemented with Canada balsam or other suitable adhesive. The top plate (c) is made from a 6-in. diameter metal disc about $\frac{1}{4}$ -in. thickness drilled with a central 1-in. hole and fitted with a $\frac{1}{4}$ -in.

diameter tube for connection to the pump brazed half-way between the centre and circumference. A rubber washer (d) forms a gasket between the plate and glass tubing and a second thick rubber washer (e) ensures an airtight junction between the filter funnel or Gooch adaptor and the metal disc. Using the above dimensions filtration is possible into graduated flasks up to 500 ml. and separators up to 250 ml. capacity. Smaller apparatus may be raised to a convenient height on wooden blocks.

NEW REMEDIES (continued from page 271)

colour with Reinecke's salt and a yellow colour with trinitrophenol and with sulphuric acid and potassium iodate. *d*-Tubocurarine chloride, dried at 100°C. for 4 hours, loses not more than 11.5 per cent. of its weight, and contains 9.5 to 10.2 per cent. of chlorine, calculated on the dried material. The weight of the residue obtained by treating an aqueous solution with sodium bicarbonate, extracting with chloroform, removing the chloroform and drying is not more than 3 per cent., calculated on the dried material, and the residue itself is insoluble in water, but soluble in dilute hydrochloric acid. *d*-Tubocurarine chloride has a specific rotation in 1 per cent. w/v solution of -208° to $+217^\circ$ (the most probable value for pure anhydrous *d*-tubocurarine chloride is $+215^\circ$). It is standardised biologically by the rabbit "head-drop" method; the standard "head-drop" dose HD50, calculated as $C_{25}H_{44}O_6N_2Cl_2 \cdot 5H_2O$, is 0.15 mg./kg. of body weight, and references to the method of assay are given. *d*-Tubocurarine chloride is used to reduce the tone or contractile power of skeletal muscle in anaesthesia, shock therapy, and in certain spastic states. It is also used for the diagnosis of myasthenia gravis.

G. R. K.

REVIEW ARTICLE

THE PHARMACOLOGY OF CURARE AND CURARISING SUBSTANCES

BY W. D. M. PATON, B.A., B.M.

National Institute for Medical Research, Hampstead, N.W.3

HISTORICAL

EIGHTY years have now elapsed since Crum Brown and Fraser^{1,2}, in a paper still worth close study, laid the foundations of our knowledge of the relation between the chemical structure and the pharmacological action of quaternary salts. Even at that early date, they referred to the therapeutic possibilities of the new compounds they had described, and to their advantages over curare in being "readily obtained in a state of perfect purity, and, therefore, of constant strength." It is only in recent years, however, that either a natural alkaloid or a synthetic curarising substance has emerged in good supply and of the required properties. This rapid progress has been greatly stimulated by King's determination of the structure of *d*-tubocurarine chloride^{3,4,5} by the success of Bennett⁶ in softening therapeutic convulsions with a curare extract prepared by MacIntyre and standardised by Holaday's rabbit head-drop method; by Griffith and Johnson's pioneer demonstrations of the usefulness of curare in anæsthesia⁷; by Wintersteiner and Dutcher's isolation⁸ of a potent curarising extract (intocostrin) rich in *d*-tubocurarine chloride, from a known botanical species (*Chondrodendron tomentosum*); and by the discovery of Bovet and his colleagues of synthetic compounds with a potency comparable to that of the natural alkaloids^{9,10,11,12,13}. As a result of this and other work, the clinician now commands a choice of reliable and well-studied compounds, and the pharmacologist has been enriched by many stimulating (and often bewildering) additions to his knowledge.

RECENT LITERATURE

Four important reviews should be consulted for detailed references: Ing has reviewed the relation of the chemical structure of onium salts to their pharmacological action in an authoritative article¹⁴. MacIntyre's "Curare"¹⁵ is a very valuable source-book, particularly on the history of the subject: Bovet and Bovet-Nitti¹² have reviewed recent work, particularly their own studies of "curares de synthèse": and Craig¹⁶ has compiled and discussed a very extensive list of compounds tested up till 1947 for curarising action. There are many reports of use in medicine, surgery and anæsthesia, for which reference should be made to the clinical journals. A recent discussion by Kuffler, Acheson, Welsh and Harvey¹⁷ of theories of neuromuscular transmission also deserves attention.

The present article is not intended to be a comprehensive survey of the subject, but attempts to review important features of the work of

recent years in the light of previous knowledge, and to indicate some of the problems remaining unsolved.

MODES OF "CURARISATION"

The term "curarisation" is no longer restricted in current use to that form of neuromuscular block caused by curare, but is sometimes used where similar effects are produced by other drugs, even where no more than muscular relaxation is meant (e.g., the action of myanesin). This implies, of course, a considerable widening of the meaning of the word, and a corresponding risk of confusion. But such extension of meaning is not altogether inappropriate; for block at the neuromuscular junction can be regarded as a special case of synaptic block, which may also occur at the ganglionic or central nervous synapse. Dale¹⁸ and Feldberg¹⁹ have discussed the evidence that the transmissions at each of these synapses possess features in common, with particular reference to the possibility of a common mechanism of chemical transmission. It should be noted that if this evidence is accepted, the failure of curarising substances to exert similar actions at each type of synapse is a fact of the first importance. For the time being, however, the interests of clarity are best served by simply describing, with particular reference to the neuromuscular junction, the different kinds of synaptic block that can be referred to as curarisation. The opportunity will be taken, in doing this, of mentioning some of the recent additions to knowledge in this field.

I. THE NEUROMUSCULAR JUNCTION. (a) *The action of curare alkaloids.* Classical experiments have established that after paralysis of a muscle by curare to excitation through its nerve, conduction in the nerve trunk is unaltered and the muscle can still give a propagated contraction in response to direct electrical stimulation. The site of the paralysis is thus localised to the nerve terminals and motor end-plate. Dale and his colleagues have further shown that the terminal nerve endings of the curarised muscle still liberate acetylcholine, and that arterially injected acetylcholine is antagonised as much as, or more than, the effect of a nerve volley. Apart from the relevance of these facts to the mechanism of neuromuscular transmission, they are also important criteria of curare-like action. To them may be added the inhibition by curare of the contraction of frog's rectus due to acetylcholine; the antagonism of anticholinesterases to the actions of curare on the neuromuscular junction; the failure of the partially curarised muscle to sustain a tetanus; and the revealing, under suitable conditions, of a transient potential at the end-plate excited by nerve stimulation, which has been closely studied and termed the "end-plate potential" by Eccles and his colleagues. These, among other characteristics, constitute a highly specific picture.

The normal conception of neuromuscular transmission at present is as follows: the wave of excitation reaches the nerve terminals, and there causes the discharge of acetylcholine in close relation to the motor end-plate. This discharge depolarises the end-plate (giving rise to the end-plate potential) and the depolarisation of the end-plate in turn excites the

muscle fibre. If this conception is accepted, then the characteristic actions of curare lend themselves very readily to the belief that curare acts by raising the threshold of the motor end-plate to excitation by acetylcholine, and that it exerts this action by competing for the acetylcholine receptor sites on the end-plate. But although this view of its action is both plausible and widely held, it cannot be said to be established beyond all question, for there are many phenomena of curarisation that still remain unexplained. It certainly provides, however, a most useful working hypothesis.

It is unfortunate that the specific tests mentioned are rarely used to verify that a substance is "curare-like"; proof of excitability of nerve and muscle is commonly omitted, and investigation of effect on acetylcholine release is a rarity. One test sometimes used, that of antagonism by anticholinesterases, is, by itself, useless, since these substances may greatly increase the tension of the twitch of the normal uncurarised muscle.

(b) *The action of bistrimethylammonium decane diiodide (C10).* This compound, which will be further discussed below, is curare-like in four respects: during its action, conduction in nerve is unaltered, direct excitability of muscle is retained, injected acetylcholine is rendered ineffective, and release of acetylcholine by motor nerve stimulation is not prevented. But there are also important differences; C10 itself elicits a contraction of frog's rectus; it is not antagonised by anticholinesterases, although C5 (the pentane homologue) is an effective antagonist; its activity varies very greatly with species of animal used for test; and it produces a depolarisation of the muscle membrane²⁰. None of these effects is shown by curare. The mode of action of C10 is still uncertain, but these and other differences from curare are sufficiently great to make it necessary to distinguish the actions of the two drugs.

(c) *The action of anticholinesterases.* Eserine has long been known as a depressant of the muscular contraction caused by a tetanus of the motor nerve, although it usually augments the tension of single twitches. This depressant action exerted both by eserine and by other anticholinesterases, is due to the accumulation of paralysing concentrations of acetylcholine at the end-plate (Brown, Dale and Feldberg²¹). It is not possible, however, to exclude entirely some direct action by the anticholinesterase itself (cf. Riker and Wescoe²²).

(d) *The action of substances depressing the release of acetylcholine.* Harvey²³ has presented evidence suggesting that some of the neuromuscular block caused by procaine is due to interference with release of acetylcholine by the nerve-ending. A similar block is caused by botulinus toxin (Burgen, Dickens and Zatman²⁴) after which progressive failure of transmission occurs, although nerve and muscle remain excitable: acetylcholine injected is still effective, but release of acetylcholine is depressed. Brown and Harvey²⁵ and Brown and Vianna Dias²⁶ have reported that such a failure of acetylcholine release also results from calcium deficiency or from injection or perfusion with solutions rich in phosphate.

2. **THE GANGLIONIC SYNAPSE.** Depression of transmission at the synapse of the superior cervical ganglion by curare, without loss of excitability of preganglionic fibres or of ganglion cells, or abolition of the release of acetylcholine has been shown by Brown and Feldberg²⁷. Brown and Feldberg²⁸ have also demonstrated block of transmission due to accumulation of acetylcholine in the presence of eserine. Harvey²³ found that procaine blocked transmission by preventing release of acetylcholine at preganglionic nerve terminals, and Harvey and MacIntosh²⁹ have shown that calcium lack in the perfusion fluid leads to the same result. Block by injection of large doses of potassium has also been described (Brown and Feldberg³⁰).

The same types of "curarisation" may, therefore, be seen in the ganglion as at the neuromuscular junction. One of the most interesting developments recently, however, has been the observation that a "curarising" compound which is active at the neuromuscular junction may be relatively inactive on the ganglion and *vice versa*. It has, of course, been known for some time that tetraethylammonium iodide, although of negligible activity at the neuromuscular junction, is a powerful paralysing agent of ganglionic transmission (Burn and Dale³¹, Acheson and Moe³²). Depierre³³ studied this point on certain of Bovet's ethyl-choline ethers of phenol and polyphenols, using the contraction of the cat's nictitating membrane excited by stimulation of the cervical sympathetic. These compounds can be arranged in a series in which curarising activity increases as ganglionic activity diminishes (so that the ratio of curarising dose to ganglion-paralysing dose ranges from *c.* 30 to 0.02). A similar dissociation occurs in the *bis*trimethylammonium series: here C5 or C6 injected intravenously into the cat requires only 1.0 mg./kg. to affect the superior cervical ganglion, but 10 to 20 mg./kg. or more are required to depress neuromuscular conduction; C10, on the other hand, active at the junction in a dose of 30 µg./kg. requires more than 3 mg./kg. to depress ganglionic transmission. The ratio just mentioned is thus more than 100 for C5 and less than 0.01 for C10. It is clear from these and similar results that activities in paralysing neuromuscular transmission and ganglionic transmission can be very widely dissociated.

3. **THE CENTRAL NERVOUS SYNAPSE.** No method yet exists adequate to determine whether a given depression of activity by the spinal cord or brain is due to paralysis of transmission at the synapse rather than to failure of conduction in neurone or axon. The action of a drug such as myanesin however, suggests some such action, since it can depress reflex activity without depressing conduction in a peripheral nerve. The point most relevant, at the moment, is the remarkable lack of central action of drugs such as *d*-tubocurarine chloride or C10. Some of this inactivity may be due to failure to pass through the capillaries of the central nervous system, which cations traverse but slowly (Krogh³⁴). But even when curare is administered more directly, it appears most commonly to exert a central stimulant action. It is possible, therefore, that the central synapse is, like the neuromuscular and ganglionic synapses, relatively specific as regards the agents which block it; we have to contrast drugs

like curare (notably paralytic on the neuromuscular junction and ganglionic synapse, but centrally stimulant); tetraethylammonium iodide (almost inactive at the junction, highly active on the superior cervical ganglion, and of mixed action on the central nervous system) (Salama¹⁰); and myanesin (inactive on neuromuscular conduction but centrally depressant).

In referring, therefore, to "curarisation" in its extended sense, it is necessary to specify both the synapse at which paralysis of transmission of excitation occurs, and the mode of that paralysis. A tentative summary of the modes of paralysis of transmission due to various drugs described can be attempted. (a) Competition block (e.g., curare) in which the threshold of the end-plate to acetylcholine is raised; (b) depolarisation block (e.g. potassium chloride and possibly ClO); (c) block by accumulation of acetylcholine (e.g. anticholinesterases); (d) block by transmitter failure, either by immobilisation of acetylcholine (e.g. procaine, calcium lack), or by deficiency of acetylcholine (e.g. botulinus toxin). This variety of modes of "curarisation" makes it essential to define clearly which is being used, even if the fundamental mechanism of the particular type of block is not fully understood. These distinctions become all the more necessary if any attempt is to be made to relate "curarising" potency to chemical structure. For the purpose of this review, the distinctions just made will not be pursued, and discussion will be confined to the pharmacology of compounds producing block of curare-like or ClO-like character.

NATURAL ALKALOIDS

1. THE CURARE GROUP. The total number of such alkaloids is very large. For practical purposes three should be carefully distinguished: (a)

OCH₃

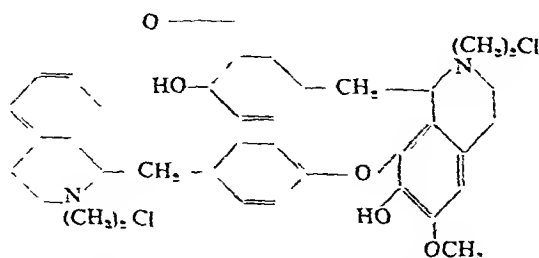


FIG. 1. Structure of *d*-Tubocurarine chloride.

curarine chloride; this is the material isolated by Boehm from calabash curare, much used in experimental work before the isolation of *d*-tubocurarine chloride: (b) *d*-tubocurarine chloride (*d*-T.C.), isolated by King¹¹ from tube curare, whose structure was finally determined by him¹²: (c) intocostin, an extract prepared by Squibbs, rich in *d*-tubocurarine chloride; the intocostin unit is equivalent to 1 mg. of a standard preparation from *Chondrodendron tomentosum*, and the activity of 6.5 units is equal to that of 1 mg. of *d*-tubocurarine chloride, on the rabbit head-drop test. "Curare" is used in this review as a generic term when the distinction between the above substances is not important.

The main features of the pharmacology of these substances at the neuromuscular junction have already been described; further details will be found in the references cited. But other properties of the compounds have recently come into prominence arising particularly with reference to the possibility of side-actions in clinical use.

(a) *Liberation of histamine.* The observation that curarine liberated histamine from muscle by Alam, Anrep, Barsoum, Talaat and Weininger³⁶ has been repeated by Gregory and Schild³⁷; the latter showed that *d*-T.C. exerted this action both on the perfused tongue of the cat and on the rat's diaphragm. Grob, Lilienthal and Harvey³⁸ have extended these results to man, showing that *d*-T.C. injected into the brachial artery causes flushing, œdema, and itching of the arm, which is lessened by anti-histamines, and that injected intradermally it causes a typical "triple response." Striking confirmation of this work was provided by Landmesser³⁹, who showed that *d*-T.C. caused fall of blood pressure in spinal dogs and bronchoconstriction of guinea-pig's lung similar to that caused by peptone, and that these effects were prevented by antihistamines. Further, he observed that occasional dogs were refractory to the effects of *d*-T.C. (just as they are to peptone), and that sensitive animals could be made refractory to peptone by previous injection of adequate doses of *d*-T.C., or *vice-versa*. *d*-T.C. thus resembles peptone rather closely, and it is highly probable that, as with peptone, the liberation of heparin accompanies the liberation of histamine by the drug. As Landmesser points out, the practical application of these results is difficult, since, for instance, anæsthetics depress the release of histamine. But it is clearly desirable, at least, that compounds less active in this respect should be investigated.

(b) *Actions on the C.N.S.* That *d*-T.C. has some central stimulant property has already been mentioned. The experimental results in this field, however, are still somewhat confused. References to the stimulant action of curare when applied directly to the central nervous system may be found in the reviews mentioned. Salama³⁵ has recently verified these results, administering *d*-T.C. directly into the ventricles of cats. It is common to see a stage of excitement preceding curarisation with *d*-T.C., particularly with smaller animals. Everett⁴⁰ has described the convulsant action of *d*-T.C. given intracisternally to rabbits. On the other hand, it also has been claimed that curare given intravenously may have a narcotic or anæsthetic action. Whitacre and Fisher⁴¹ report an illustrative case from their surgical experience. The report by Prescott, Organe and Rowbotham⁴², however, and the careful and courageous experiment by Smith, Brown, Toman and Goodman⁴³ fail to substantiate this. In the latter paper, an account is given of the curarisation of a volunteer, under artificial respiration, so deeply that not even the most trivial muscular movement could be made; nevertheless, a full and intelligent narrative of his experience was furnished by the subject after recovery. Kellgren, McGowan and Wood⁴⁴ found no alteration in sensation after small doses of *d*-T.C. Paton and Zaimis⁴⁵, studying

the respiratory depression by *d*-T.C., found that the discharge down the phrenic nerve of the cat was not depressed by an intravenous dose of *d*-T.C. sufficient to abolish spontaneous respiration. It seems unlikely from this and other work, therefore, that the central actions of *d*-T.C. are important after intravenous doses, probably because (as mentioned above) it would not be expected to pass very readily through the capillaries of the central nervous system.

(c) *Action on autonomic ganglia.* Curare has long been known to depress ganglionic transmission, as has been already mentioned. Such action has, indeed, been suggested as the basis of an assay method, in which inhibition of the peristaltic reflex of isolated intestine is used to assay *d*-T.C. or kindred drugs (Feldberg and Lin⁴⁶). Gross and Cullen⁴⁷ have shown that in the dog, curarising doses of intocostin or *d*-T.C. cause inhibition of peristaltic activity by stomach and small intestine, with some loss of tone; fall of blood pressure was also sometimes observed. Heymans^{48,49} found that rapid injections of intocostin cause a fall of blood pressure and depression of the cardiovascular reflexes, but that with slow injection of the same dose these actions did not appear. Prostigmine did not abolish these effects.

(d) *Antagonism by certain dyes.* An old observation that certain dye-stuffs antagonise curare has been reinvestigated by Kensler⁵⁰. Congo red, chlorazol fast pink and Evans Blue are highly effective at both preventing and relieving paralysis of frogs by *d*-T.C. The action is due to the formation of a precipitable complex, in which form *d*-T.C. is not active. The phenomenon promises to be a useful tool in suitable circumstances.

(e) *Anticholinesterase action.* Some of the earlier preparations of intocostin contained material with an appreciable power of inhibiting cholinesterase (Harris and Harris⁵¹). Pure *d*-T.C., however, has slight activity in this respect; the materials responsible were tertiary bases of negligible curarising activity. The finding is of interest, in view of the extent to which anticholinesterase action has been observed among synthetic compounds.

2. DIMETHYL ETHER OF *d*-TUBOCURARINE CHLORIDE. It has been known for some time that methylation of *d*-T.C. increases its potency. Further studies (Collier, Paris and Woolf⁵²) have shown that the dimethyl ether is about 10 times as active as *d*-T.C. in rabbits, and that it displays certain species differences in potency and duration of action. In the main, it is very similar to *d*-T.C., but considerably more active. Successful clinical trials have been reported by Stoelting, Graf and Viera⁵³.

3. THE ERYTHRINA ALKALOIDS Exceptional interest attaches to these compounds, of which erythroidine (from the seeds of the legume *Erythrina Americana*) and β -dihydroerythroidine (obtained by hydrogenating erythroidine) are the most important. (Erythrina extracts are said to have been used in the treatment of convulsions as long ago as 1887.) They are at present unique in being highly active and yet possessing only trivalent nitrogen atoms; on converting the latter to quaternary nitrogen, the compounds diminish greatly in potency. So far as their

action at the neuromuscular junction is concerned, they resemble the curare alkaloids, having 1/5th or less the activity of *d*-tubocurarine chloride, and they are antagonised by anticholinesterases. In other respects, there are important differences; the erythroidines are active by mouth; they do not possess anti-esterase activity (Harris and Harris⁵¹); they do not share the ability of *d*-tubocurarine to liberate histamine (Landmesser³⁹); they are not antagonised by congo red (Kensler⁵⁰); they possess a feeble atropine-like action. Clinical trial has been reported (Harvey and Masland⁵⁴, Dripps and Sergent⁵⁵); the most serious disadvantage appears to be a depression of the blood pressure with effective doses; respiratory depression is also common. It is to be hoped that other members of this series may be discovered which are free from these defects.

4. OTHER NATURAL ALKALOIDS AND THEIR DERIVATIVES. The comprehensive study of quinine methochloride by Harvey⁵⁶ requires mention; this is one of the few compounds investigated whose action on the release of acetylcholine has been tested; in paralysing doses, it fails to prevent such release in the superior cervical ganglion of the cat. It is curare-like in most respects, is about 1/40 times as active as *d*-T.C., and is active by mouth. Trials in man have been reported by Harvey and Masland⁵⁴. A large number of other related alkaloids of the cinchona group have also been studied but are not promising clinically.

Among compounds related to *d*-T.C., the isochondrodendrines (Marsh and Pelletier⁵⁷) and the chondrodendrines (Marsh, Sleeth and Tucker⁵⁸) have been investigated; in both groups variation of potency with species, and increase in potency by methylating free hydroxyl groups were found, analogous with the effect of methylating *d*-tubocurarine chloride. Another related compound, N-methyl oxyacanthine (Marsh, Herring and Sleeth⁵⁹) is of interest, since in man it lessens salivary secretion, and has a weak atropine-like action (antagonising the depression of dog's blood pressure by acetylcholine).

The most potent known curarising substances are the toxiferines, isolated by Wieland from calabash curare; they are effective in doses of the order of 10 µg./kg. in frogs and rabbits. So far as is known, however, they do not appear to be sufficiently free from side-actions of various kinds to be suitable for therapeutic use.

On the whole, therefore, only two serious rivals to *d*-tubocurarine chloride have emerged from the natural alkaloids and their derivatives—the methyl ether of *d*-T.C. itself, and the β-erythroidines. Apart from questions of potency the compounds do not differ significantly from curare, and do not, for instance, eliminate the need for careful control of respiration. Information is not yet adequate to assess their side-actions in clinical use.

From the pharmacological side, *d*-T.C. is clearly not the ideal curarising agent, because of its ganglionic action and its power of liberating histamine. Its depression of the respiratory minute volume is probably less important, since modern methods of anaesthesia are fully adequate to maintaining artificial respiration without inconvenience. A

technical disadvantage of it is that some preparations cannot be given simultaneously with pentothal owing to mutual precipitation. Finally, it is a drug that is relatively expensive and difficult to prepare in the pure state.

Against all this, it is important to realise that it is with this compound that the use of curarising substances in anaesthesia has established itself; this rapid success, and the widespread search for substances of similar action are, indeed, sufficient testimony to its value.

SYNTHETIC CURARISING SUBSTANCES

1. **EARLY WORK.** The first synthetic compound exhibiting curare-like activity, tetramethylammonium iodide, $(N(CH_3)_4)_2I_2$ was made by Crum Brown and Fraser² in 1869, and provided them with a striking confirmation of their theories. Its curariform action is not strong and its power of stimulating autonomic ganglia is its most prominent action in the cat, followed by a weaker paralysing action on ganglia; it also possesses appreciable muscarine-like action (Burn and Dale³¹). Bacq and Brown⁶⁰ found that it could also elicit a contraction from mammalian striated muscle, and it is known to cause a contracture of frog's rectus. It is typical of the many related compounds that they possess, besides curariform activity, some or all of these other activities in some degree—some of them also possessing anticholinesterase potency. But none of the synthetic monoquaternary salts, whether simple tetralkylammonium halides or choline or betaine derivatives, proved to be sufficiently potent or free from side-actions even to approach the natural alkaloids.

2. **BOVET'S COMPOUNDS.** The work of Bovet and his colleagues marked an important advance. Taking as a model the structure of *d*-tubocurarine chloride (which had been almost completely defined by King at that time).

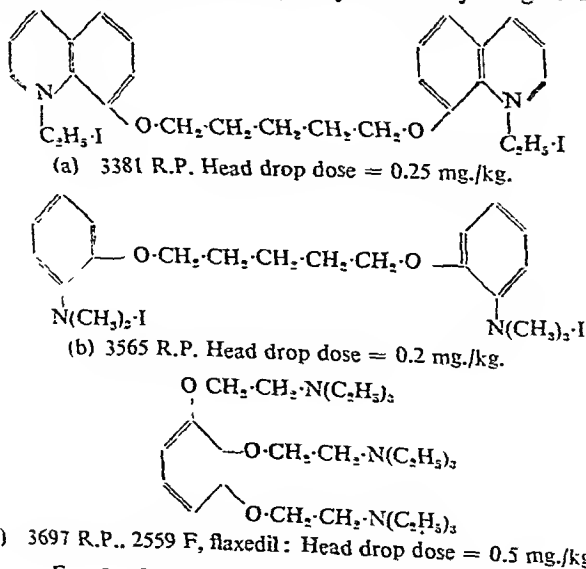


FIG. 2. Synthetic curarising compounds (Bovet).

simpler related structures were synthesised. Using this basic structure, variations of chain-length and quaternary substituents were studied. The first compound reported (Bovet, Courvoisier, Ducrot, and Horclois⁹), 3381 R.P., is also the first synthetic compound that resembles curare at all closely, in being potent and sensitive to anticholinesterases (Fig. 2(a)). It possesses, in addition, some anticholinesterase potency (Halpern, Benda and Bourdon⁶¹).

The next step was a further simplification of structure, as a result of which 3565 R.P. (see Fig. 2b) was described (Bovet, Courvoisier, Ducrot and Horclois¹¹). This compound, too, is highly active, but does not possess anti-esterase action; it is antagonised by anti-esterases, and has some nicotinic action.

The third main series were the choline ethers of phenols and polyphenols (Bovet, Depierre and Lestrang¹⁰), of which the ethyl-choline triether of pyrogallol proved to be the most important (2559 F. or 3697 R.P., flaxedil. See Figure 2c). This possessed much the same actions as the other compounds with fewer side-actions.

Finally, an interesting series of choline esters has been built, also of considerable potency, but of transient action (Bovet, Bovet-Nitti, Guarino and Fusco¹³). Figure 3 gives a comparison of one of these with the corresponding ether and the directly substituted compound, together with their effective doses and duration of action.




	<i>Head drop dose</i>	<i>Duration of paralysis after 10 H. D. doses</i>
 $\text{COOCH}_2 \cdot \text{CH}_2 \cdot \text{N}(\text{C}_2\text{H}_5)_3 \cdot \text{I}$	3 mg./kg.	10 minutes
 $\text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{N}(\text{C}_2\text{H}_5)_3 \cdot \text{I}$	3 mg./kg.	1 hour
 $\text{CH}_2 \cdot \text{CH}_2 \cdot \text{N}(\text{C}_2\text{H}_5)_3 \cdot \text{I}$	3 mg./kg.	3 hours

FIG. 3. Variation of duration of action with chemical structure.

Courvoisier and Ducrot⁶² have shown that flaxedil (2559F) possesses little power to liberate histamine, although 3381 R.P. and 3365 R.P. are active in this respect. Depierre³³ has shown that it possesses little ganglionic action.

3. THE *bis*TRIMETHYLAMMONIUM SERIES. The most potent synthetic compound so far described is the decane derivative (C10) of an even simpler series, the *bis*trimethylammonium polymethylene salts. The curarising action of the series was reported independently by Barlow and Ing⁶³ and by Paton and Zaimis⁶⁴, and the latter authors have studied the pharmacological actions of the series in considerable detail (Paton and Zaimis^{64,65}). Some of these actions of C10 have been already mentioned above, and will not be further discussed. But three points require further discussion.

(a) *Variation of potency with species and test object.* C10 is highly active in cat, 0.03 mg./kg. causing full neuromuscular paralysis; in rabbit 0.1 mg./kg. is required; in mouse 1 mg./kg., and in rat 5 mg./kg. In man a total dose of 3 mg. usually causes almost complete paralysis, and man thus corresponds very closely to the cat in sensitivity (Organe, Paton and Zaimis⁶⁵); the equivalent dose of *d*-T.C. in a man is about 15 mg.

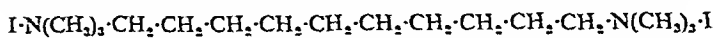


FIG. 4. Structure of *bis*Trimethylammonium decane diiodide (C10)

The comparison of *d*-T.C. with C10 further varies with the muscle used, and with the rate of stimulation. In the cat, using single twitches every 10 sec., C10 is 10-20 times as active as *d*-T.C. But if tetani are used this figure is at least halved, since *d*-T.C. depresses a tetanus much more than a twitch and C10 allows a tetanus to be fairly well sustained. In addition, the two compounds differ in their actions on different muscles; thus, in the cat, C10 paralyzes tibialis more readily than soleus, while *d*-T.C. does the reverse. Finally, a test method such as that used by de Jalon⁶⁶, in which the antagonism of curare to acetylcholine on frog's rectus is employed, cannot be used at all, since C10 itself elicits a contracture and (by reason of its anti-esterase activity) actually potentiates acetylcholine. The findings on species difference are paralleled in other compounds. Collier, Paris and Woolf⁶² have reported a species difference with the dimethyl ether of *d*-T.C., and Wien⁶⁷ has done so, using the isolated diaphragm, for some of Bovet's compounds. From such results, it is obvious that investigations of new compounds should be made by several test methods, and that in an assay the method used should be fully sensitive to all the substances likely to occur in the material under test. The fact that man closely resembles the cat in his sensitivity to C10 is an important argument for the use of cats at an early stage in any investigation. The species difference, and the different action on different muscles are fascinating problems for which there is no explanation at present.

(b) *Effect on respiration.* One of the remarkable features of the action of C10 in the cat is the failure to depress respiration significantly at a time when tibialis twitch is more than 95 per cent. paralysed (Paton and Zaimis⁶⁵). This appears to be due to the fact that tetani are well-sustained, and that red muscles are less affected than white. The contrast with *d*-T.C. is sharp, for in the cat respiration may be depressed before any action on the twitch has appeared. But it is difficult to predict

the same sparing of respiration in relation to other muscular activities (e.g., abdominal relaxation in anæsthesia or the softening of therapeutic convulsions), in the absence of information about the physiological characteristics of the muscles involved and of the rate at which their motor units are excited.

(c) *Side actions.* Curarising compounds may depress the blood pressure by liberating histamine, by paralysing ganglia or by exerting a muscarine-like action. Curarising doses of C10 have no effect on the blood pressure of the anæsthetised cat, and large doses (at least 100 times the effective curarising dose) must be given to show such an action. It is at least 5 times less active in releasing histamine or causing ganglionic depression than the same weight of *d*-T.C., and its muscarine action is negligible. C10 has some anticholinesterase action, but this does not appear to cause any undesirable effect. In man, there is no interference with sensation or consciousness after an intravenous dose of C10 sufficient to cause almost complete paralysis (Organe, Paton and Zaimis⁶⁵).

DISCUSSION

There is much that is confusing in recent developments, which cannot be discussed here. But one feature may be stressed; this is the remarkable diversity of actions among the various compounds studied. Their variation in activity on different species, on different muscles, and on different synapses; their differences in side-actions, and their wide disparity in chemical structure:—all these are hopeful prognostics of yet other compounds with useful selective actions. Before these are likely to be discovered, however, fundamental work must be done on the reasons for these diversities of action, about which we know little, and such fundamental research is the most urgent need. There are, further, many more practical requirements to be satisfied, such as a satisfactory method of prolonging the action of these drugs; compounds active by mouth and safe to use; or an antagonist to the activity of curarising agents which is free of side-actions.

To the pharmacologist, however, the most absorbing question remains that of the relation of the structure of these compounds to their pharmacological action. No field has proved more hazardous than that of the onium salts, in which to venture generalisations. Many of the important anomalies are reviewed by Ing¹⁴. Certain broad statements, however, can be made.

(1) Crum Brown and Fraser's generalisation of 1868 still remains remarkably true. The only important exception to it is that of the properties of the *Erythrina* alkaloids; and in ignorance of their structure it is impossible to say how much of an exception they represent. There is little doubt that the most promising approach, in devising substances of curarising potency, is still by way of quaternary salts; and among these, there is still no rival to the salts of quaternary nitrogen.

(2) The introduction of a second or even a third quaternary group into the molecule appears to be an important element in producing compounds of high curarising potency: all the compounds of known

chemical structure active in a dose of 1 mg./kg. or less have two or more such groups. Possible reasons for this enhancement of potency are not far to seek in the favourable effects such additions have on the attachment of the drugs to a receptor surface.

(3) The introduction of further quaternary groups has another effect: the monoquaternary compounds active on the neuromuscular junction are commonly like tetramethylammonium iodide, particularly in possessing stimulant nicotine-like and muscarine-like actions. In bisquaternary salts these actions are replaced by weak anti-esterase activity, ganglionic depression, and histamine liberation. These latter actions would, indeed, be a serious disadvantage: but bisquaternary or terquaternary compounds have been obtained in which they are slight, although the compounds still possess high potency (C10 and flaxedil).

(4) A further significant point appears to be the spatial separation of the two quaternary groups. It can hardly be coincidental that in such active compounds as *d*-tubocurarine chloride and its methyl ether, Bovet's compounds 3381 R.P. and 3565 R.P., and C10, the quaternary groups should be separated by 10 to 11 atoms, particularly since shortening the chain in the bistrimethylammonium series to less than 7 carbon atoms almost completely abolishes activity. The implications of this require further study, but the suggestion certainly appears that the ability to interfere with neuromuscular transmission depends not only on certain characteristic polar groups but also on their characteristic spatial location. It is certain that other considerations than distance are also concerned; thus, King⁴ reports that *l*-tubocurarine chloride is 30 to 60 times weaker than the dextro-rotatory isomer.

Finally, it is worth pointing out that the diversity of structure exhibiting curarising activity suggests that the specific characteristics of such compounds differ in some important way from those of (for instance) muscarine-like substances. Pfeiffer⁶ has drawn some striking analogies between the distances separating certain prosthetic groups (the nitrogen atom and two oxygen atoms) in muscarine-like and atropine-like substances, and the distances between the same groups in acetylcholine. But no such analogy is at present visible among curarising substances. It is, indeed, possible that their specific activity is not dependent solely on a relationship to acetylcholine, but that resemblance to some other physiologically active cation must be considered. The papers of Ing and Wright still represent the most critical approach to the subject; recent attempts to apply modern concepts of atomic structure to the problem (Holmes, Jenden, Taylor⁷) do not advance much beyond the position of Ing and Wright.

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RESEARCH PAPERS

THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART I

THE LIBERATION OF PHOSPHORUS AS PHOSPHATE FROM VITAMIN B₁₂ BY ACID HYDROLYSIS

By B. ELLIS, V. PETROW AND G. F. SNOOK

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WORK in these laboratories on the anti-pernicious anæmia factor present in liver has led to the isolation from anahæmin¹ of a clinically active red crystalline compound which we have characterised by:—

(i) lack of a definite melting-point, the crystals darkening at 190° to 250°C.

(ii) an R_F value of 0.1 when run on unidimensional paper-strip partition chromatograms using *n*-butyl alcohol as a solvent.

(iii) characteristic light absorption, an aqueous solution showing maxima at 550 m μ (shoulder at ca. 520 m μ), 361 m μ , and 278 m μ , with inflections at 322 m μ and 304 m μ .

(iv) the presence of nitrogen, phosphorus and cobalt in the molecule (see also Rickes *et al.*²; Smith³).

(v) a cobalt content of 4.0 per cent for material dried *in vacuo* at 76°C.

(vi) the presence of one ninhydrin-reacting substance detected in hydrochloric acid hydrolysates run on paper-strip partition chromatograms.

The identity of our compound with the substance to which the name vitamin B₁₂ was first applied⁴ has now been established by a direct comparison with an authentic specimen, kindly carried out for us by Merck Laboratories, Inc., through the courtesy of their Vice-President and Research Director, Dr. Randolph T. Major.

More detailed examination of the paper-strip chromatograms obtained in (vi) above revealed the presence of phosphate on them, an observation which led us to undertake a quantitative study of the liberation of this ion from vitamin B₁₂ during its hydrolysis with hydrochloric acid. The solutions obtained on hydrolysis, however, are deeply coloured owing to the survival of a pigmented cobalt-containing fragment of the B₁₂ molecule to which we have already referred in an earlier publication¹. The problem of estimating phosphate liberated on hydrolysis thus resolved itself into a search for a micro-method which would permit quantitative separation of the inorganic ion from the pigmented material both of which are present in the hydrolysate. We ultimately achieved this by a new application of the unidimensional paper-strip chromatography technique. In addition, we have carried out some exploratory experiments on the behaviour of

phosphates on chromatograms irrigated with a number of solvents, the results being reported below (Experimental (i)).

The method finally adopted for the estimation of phosphate consisted in spotting the vitamin B₁₂ hydrolysate on to strips of filter-paper, irrigating with isobutyric acid, locating phosphate on the guide strips by means of the ammonium molybdate and benzidine spot test reagents⁵, and estimating the phosphate colorimetrically after its elution from the paper.

We were unable to obtain evidence for the liberation of phosphate from vitamin B₁₂ when the compound was hydrolysed with 20 per cent. hydrochloric acid at room temperature for periods as long as 17 days. Phosphorus must therefore be present in a fairly stable form. At 100°C., however, liberation of phosphorus as phosphate took place and appeared to be essentially complete after about 6 hours, values corresponding to a phosphorus content in vitamin B₁₂ of *ca.* 2.0 per cent being obtained. This was equivalent to all the phosphorus present in vitamin B₁₂, as total phosphorus determinations gave results ranging between 1.9 and 2.3 per cent. of phosphorus.

We have previously reported that vitamin B₁₂ contains 4.0 per cent. of cobalt¹. This figure, taken in conjunction with a phosphorus content of 2.1 per cent., shows that the atomic ratio cobalt:phosphorus in the vitamin B₁₂ molecule is 1:1. The anti-pernicious anæmia factor isolated by Smith and Parker⁶, it may be added, is reported by Smith^{3,7} to have a cobalt:phosphorus ratio of 1:3. It follows from this that the compound described by Smith and Parker⁶ must differ from vitamin B₁₂, a conclusion supporting the view that at least two hæmatopoietic factors containing cobalt may be obtained from liver. Further work is required to establish whether in fact both these compounds co-exist in liver or whether, as is possible, one of them is a clinically-active artefact produced from the other during the process of isolation.

EXPERIMENTAL.

Whatman No. 1 filter paper was used for all chromatograms. Solvents used for irrigation were saturated with water, with the exception of isobutyric acid which was employed as a 65 per cent. aqueous solution. Phosphorus was estimated colorimetrically by the method of Fiske and Subbarow⁸. A "Spekker" photoelectric absorptiometer with an Ilford Spectrum filter No. 608 was used throughout for colour intensity comparisons, in conjunction with at least two phosphate standards. These were freshly prepared on each occasion and gave absorptiometer readings satisfactorily coinciding with points on a previously constructed calibration curve.

(i) *Detection, behaviour, and quantitative estimation of the phosphate ion on paper-strip chromatograms.* Disodium hydrogen phosphate, potassium dihydrogen phosphate, diammonium hydrogen phosphate, and phosphoric acid were employed as sources of phosphate ions. Phosphate was readily detected on the chromatograms using the ammonium molybdate and benzidine reagents employed in spot-test techniques⁵. For this purpose, the chromatogram, after irrigation with the solvent, was dried

and lightly sprayed with a reagent consisting of a solution of 5 g. of ammonium molybdate in a mixture of 100 ml. of water and 35 ml. of concentrated nitric acid. The presence of phosphate on the paper, in quantities exceeding 2 μ g. of phosphorus, was indicated by the appearance of a yellow spot or zone, which changed to blue after spraying with a solution prepared from 50 mg. of benzidine (or benzidine hydrochloride) in 100 ml. of 10 per cent. acetic acid, followed by exposure of the area to ammonia vapour. Diffusion or running of the coloured spot or zone was minimised by spraying very lightly with the two reagents, whilst excessive exposure to ammonia was avoided as this tended to reduce the intensity of the blue colour.

n-Butyl alcohol, isobutyric acid, collidine, and phenol were employed as solvents in the present investigation.

n-Butyl alcohol. Migration of phosphate did not occur.

*iso*Butyric Acid. Slightly elongated spots falling within the region R_F 0.20 to R_F 0.25 were obtained with phosphoric acid and with all the phosphates. The diammonium hydrogen salt gave, in addition, a second smaller spot at R_F 0.3.

Collidine. Elongated tapering zones, extending from the source to points determined by the nature of the cations present were obtained. The phosphoric acid and diammonium hydrogen phosphate zones were longest, terminating at R_F 0.20, whilst those formed by the potassium and disodium salts terminated at R_F 0.17 and R_F 0.13, respectively.

Phenol. The major portion of each phosphate migrated as a spot to *ca.* R_F 0.1, but a small fraction resisted migration and remained as an annular ring at the source. It was important that all traces of phenol be removed from these chromatograms before application of the ammonium molybdate reagent, otherwise the chromatograms turned black or brown.

Experiments directed to the quantitative estimation of phosphate were limited to chromatograms irrigated with isobutyric acid as, when used for the chromatography of acid hydrolysates of vitamin B₁₂, complete separation of the phosphate and pigmented material occurred.

The technique used for locating phosphate and preparing "cuts" for elution followed that described by Consden, Gordon and Martin⁹. Twenty microlitres of an aqueous solution containing a known amount of phosphate (equivalent to 10 to 20 μ g. of phosphorus) were distributed on 8 spots, 1 cm. apart, along a starting line drawn 6 cm. from one end of a paper strip 12 cm. wide. After irrigation of the paper with isobutyric acid a "cut" containing the phosphate was trimmed to a point at one end and eluted by the method of Dent¹⁰. Phosphate in the eluate was estimated colorimetrically by the method of Fiske and Subbarow⁸. Substantially quantitative recoveries (96 to 102 per cent.) were obtained in all of 14 estimations using both phosphoric acid and its salts.

In current paper-strip chromatography technique, acid hydrolysates are evaporated to dryness to remove hydrochloric acid and the residues dissolved in distilled water before being spotted on the paper strips. In quantitative work such a procedure involves the risk of errors arising during evaporation and re-solution. In order to establish whether this

procedure could be eliminated during estimation of phosphate in acid hydrolysates of vitamin B₁₂, control experiments were carried out in which phosphoric acid dissolved in 20 per cent. hydrochloric acid was spotted directly on the paper strips. The spots so obtained were allowed to dry at room temperature for at least 1 hour before irrigation with isobutyric acid. Chromatograms prepared in this way showed fairly uniform areas of waterlogging within which, however, the phosphate could be located in approximately its normal position. Elution, followed by estimation, resulted in substantially quantitative recoveries as before. Some degree of waterlogging could therefore be ignored in these estimations. It followed from this that acid hydrolysates of vitamin B₁₂ could be employed directly for phosphate estimations and that the usual procedure involving removal of hydrochloric acid by evaporation and re-solution of the residues was unnecessary.

(ii) *The liberation of inorganic phosphorus from Vitamin B₁₂ during hydrolysis with hydrochloric acid.* Sealed tubes containing 3 to 3.5 mg of vitamin B₁₂ dissolved in 200 microlitres of 20 per cent. hydrochloric acid were, with one exception, heated at 100°C. for increasing periods of time, cooled to room temperature, and opened. Twenty microlitres of each hydrolysate were withdrawn with a micropipette and dispensed on to a paper strip (12 cm. wide) in a series of 8 spots. An additional 2 to 3 microlitres of solution were placed near one edge of the paper to serve as a "guide" for the location of phosphate. The papers were irrigated overnight with 65 per cent. isobutyric acid and air-dried. The phosphate was located by means of the guide strip and fell within the region R_F 0.2 to R_F 0.3, well removed from the pigmented zones which appeared further down the chromatograms. The area containing the phosphate was cut out, eluted, and phosphorus determined colorimetrically⁸. The main results of duplicate phosphorus estimations obtained from pairs of chromatograms arising from each hydrolysate are given in the table below.

Experiment	(a) Phosphorus content of vitamin B ₁₂ per cent.	Time of hydrolysis	Temperature	(b) Phosphorus liberated as percentage of vitamin B ₁₂ used	Ratio (b)/(a)
1	1.9	17 days	18-23°	Nil	0
2	2.1	1 hour	100°	0.6	0.29
3	2.1	2 hours	100°	1.0	0.48
4	2.3	3 "	100°	1.7	0.74
5	—	6 "	100°	1.9	—
6	1.9	12½ "	100°	2.0	1.05
7	2.0	25 "	100°	2.1	1.05

(Specimens of vitamin B₁₂ used in experiments 1, 5, 6 and 7 were dried *in vacuo* at room temperature. Crystals dried *in vacuo* at 76°C. were employed in experiments 2, 3 and 4.)

Total phosphorus was estimated by dispensing aliquot portions of each hydrolysate on to small pieces of filter paper and determining phosphorus on them by the perchloric acid digestion method of King¹¹. Colour intensities, which reached a maximum 20 minutes after the addition of the aminonaphtholsulphonic acid reducing agent, were measured with a

ANTI-PERNICIOUS ANÆMIA FACTORS

"Spekker" photoelectric absorptiometer as before. These estimations were carried out in triplicate, the mean results being given in the above table.

SUMMARY AND CONCLUSIONS

1. Phosphate is found in acid hydrolysates of vitamin B₁₂.
2. Such hydrolysates are deeply coloured and, in order to determine phosphate in them colorimetrically, it has been necessary to develop a new application of the unidimensional paper-strip partition chromatogram technique.
3. The atomic ratio of cobalt:phosphorus in vitamin B₁₂ is found to be 1:1.
4. From a comparison of this result (3 above) with the values recorded by Smith³ for the anti-pernicious anæmia factor of Smith and Parker⁶, it is concluded that the latter compound is not identical with vitamin B₁₂.

The authors thank the Directors of The British Drug Houses, Ltd., for permission to publish these results.

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THE PHARMACOGNOSY OF RAUWOLFIA

By T. E. WALLIS AND S. ROHATGI

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INTRODUCTION

THE leaves and roots of *Rauwolfia serpentina* Benth. (*Ophioxylon serpentinum* Linn.), family Apocynaceæ, are mentioned in Sanskrit works describing Ayurvedic medicines under the name "sarpagandha" and are commonly known in India by the Hindustani name of "choota chand." This plant is indigenous to India, Burma, Malaya, Siam and Java, and the drug derived from it (hitherto described as root, but actually consisting of root and rhizome mixed) has been in use in indigenous medicine for several centuries in India. Its reputed successful use has attracted the attention of numerous writers on Indian Materia Medica, including Dymock¹, Watt², Chopra³, Nadkarni⁴, Khorl and Katrak⁵.

HABITAT

The plant is described^{6,7} as a large climbing or twining shrub, found in the foot-hills of the Himalayas and in the neighbouring plains from Sirhind and Moradabad to Sikkim. It occurs in Assam, Pegu, Tenasserin (at altitudes up to 4,000 feet), in the Deccan peninsula along the ghats to Travancore and in Veylong. It is also widely distributed in the Malaya peninsula and in Java.

CONSTITUENTS

In 1931, Sen and Bose⁸, in Calcutta, isolated from the root two alkaloids having different melting-points. Siddiqui and Siddiqui⁹ in the same year, working in Delhi, found five alkaloids and arranged them in two groups, which they named the ajmaline and the serpentine groups respectively; Van Itallie and Steenhauser¹⁰ confirmed these findings in 1932. Other constituents in the roots are oleo-resins, sterols, unsaturated alcohols, oleic acid, fumaric acid, glucose, sucrose, an oxymethylantraquinone derivative, a fluorescent substance and mineral salts. J. C. Gupta *et al.*¹¹ state that the roots contain about 1.21 to 1.36 per cent. of total alkaloids, and that a standardised alcoholic extract containing 0.5 per cent. of total alkaloids is being made commercially.

PHARMACOLOGICAL ACTION

According to Siddiqui and Siddiqui⁹, the alkaloids of the ajmaline group, as tried on frogs, act as general depressants to the heart, respiration and nerves; while those of the serpentine group paralyse the respiration and depress the nerves, but stimulate the heart. Sen and Bose⁸ found from their experiments on cats that the alkaloids isolated by them brought about a slight fall in blood pressure and the respiration was slightly stimulated. The heart muscle was depressed and the plain muscle, like that of the intestines, uterus, etc., was relaxed. None of these actions represents the characteristic sedative and hypnotic effect for which the drug is valued. For this reason Gupta *et al.*¹¹ in 1947,

working in India, reinvestigated the drug, giving their attention more especially to the pharmacological action of the oleo-resins present in the root. They report that these oleo-resins possess the specific sedative and hypnotic action of the drug which the alkaloids failed to produce.

DESCRIPTION

A description of the root, with brief notes upon its microscopical structure, was given in Dymock's "Materia Medica of Western India."



FIG. 1.—*Rauwolfia serpentina* Benth. (= *Ophioxylon serpentinum*, Linn.). Drawing of a flowering plant (about $\frac{1}{2}$ natural size) from Wight, *Icones plantarum Indiae orientalis*, 1840-53, plate 849. The flowers are in cymes; they have shining red pedicels and calyces, and pure white corollas. The fruits are black berries.

2nd edition, as long ago as 1885. Quite recently the root has been included in the Indian Pharmacopœial List of 1946, where the drug is represented by a brief monograph containing particulars giving information somewhat similar to that in the earlier description of Dymock. These accounts appear to be the only two available descriptions of the structure of the root. It is, moreover, obvious that the data given are not sufficient to characterise the drug properly, either in the unground condition or in the form of powder; also no reference is made to the presence of rhizome in the drug. In view of its promising therapeutic value and increasing importance as an article of commerce, it was decided to make a systematic pharmacognostical study of the drug.

MATERIAL

Four samples from different sources were examined, viz.:—

1. Sample from the Museum of the Pharmaceutical Society of Great Britain. (July, 1947, S. K. Crews.)
2. Sample from a drug supplier in Saharanpur, U.P., India, obtained in December, 1947.
3. Sample received from the drug market in Calcutta in November, 1947, the identity of which was confirmed by S. N. Bal, Curator, Botanical Survey of India, Calcutta.
4. A fresh plant with the rhizome and a few fibrous roots attached, collected from the foot of the Himalayas near Saharanpur in December, 1947, by a drug dealer. This plant was examined by Dr. Chatterji, in charge of the Indian section of the Herbarium at the Royal Botanic Gardens, Kew, and was confirmed as belonging to the family Apocynaceæ and agreeing in all particulars with the specimen of *Rauwolfia serpentina* Benth. in the Herbarium at Kew.

MACROSCOPICAL CHARACTERS

Rauwolfia roots (Fig. 2, A) occur in commerce in pale brownish-grey pieces about 4 to 10 cm. in length and 4 to 17 mm. in diameter, cylindrical or slightly tapering, rather tortuous, rarely branched, with occasional small oval or rounded scars of rootlets, usually in a tetrastichous arrangement; the rootlets themselves are very few and when present are broken off short, their diameters varying from 0.5 to 1 mm.; outer surface of the root dull, with somewhat coarse and irregular longitudinal ridges; the outer layers of the cork are soft and tend to scale off from the harder somewhat prominently ridged, yellowish-brown inner bark; patches of entire bark sometimes exfoliate exposing a hard, compact, pale yellowish wood which has a fairly smooth surface and a low density (about 0.3); occasionally pieces of aerial stem or rhizome occur attached to the root. A smoothed transversely cut surface (Fig. 2, B) shows a large pale-yellowish wood, which is compact and finely radiate and usually shows 3 to 8 growth rings. The xylem itself is very finely porous and the woody core occupies about 4/5th of the diameter of the root; surrounding the wood is a narrow yellowish-brown bark about 0.5 to 2 mm.

wide; the root is starchy throughout and the fracture is short. The drug is almost odourless and has a bitter taste.

Pieces of the rhizome closely resemble the root, but are less uniform in diameter; they are somewhat knotty and tortuous. They are best

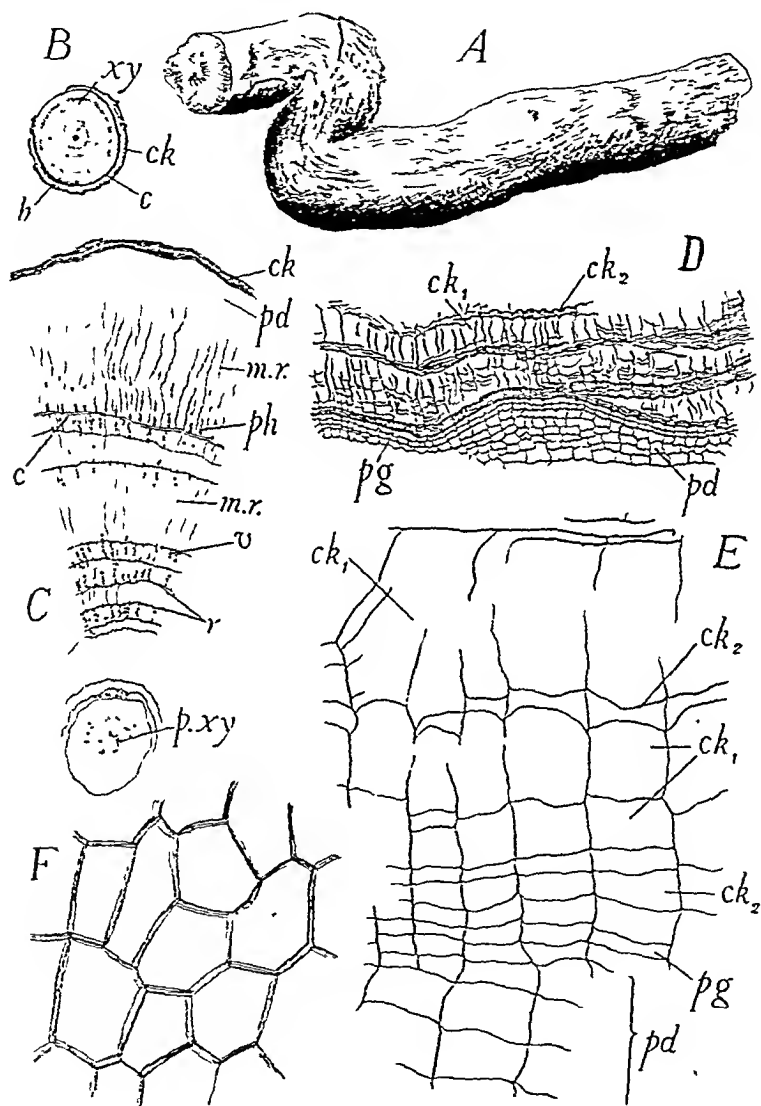


FIG. 2.—*Rauwolfia serpentina* Benth. Root. A, habit sketch of a piece of root $\times 1$. B, smoothed transverse surface of root $\times 1$. C, diagrammatic transverse section $\times 8$. D, transverse section of the tegumentary tissues $\times 40$. E, a portion of D to show details of the cells $\times 200$. F, cork in surface view $\times 200$. b, bark; c, cambium; ck, cork; ck₁, wide cork cells; ck₂, narrow cork cells; m.r., medullary ray; pd, phelloderm; pg, phellogen; ph, phloem; p.xy., primary xylem bundle; r, growth ring; v, vessel; xy, xylem.

distinguished by the smoothed transversely cut surface which exhibits a central pith having a very small diameter (about 1 to 2 mm.).

HISTOLOGY

Root: Externally there are several layers of approximately polygonal-tubular cork cells (Fig. 2, D, E and F) of two kinds, occurring in layers which in transverse sections form bands alternating with each other. One type of band consists of 1 to 5 layers of narrow cells with suberised but unligified walls measuring about $*R = 7$ to 11 to 18μ , $L = 18$ to 35

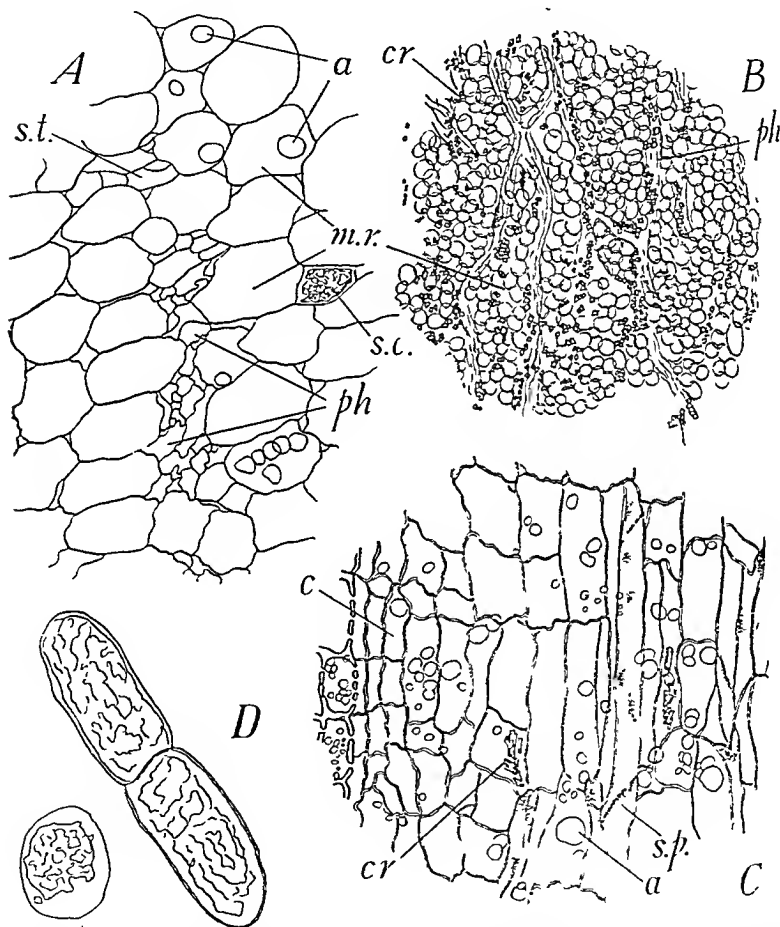


FIG. 3.—*Rauwolfia serpentina* Benth. Root. A, transverse section of the phloem $\times 200$. B, tangential longitudinal section of the phloem $\times 40$. C, radial longitudinal section of the phloem and cambium $\times 200$. D, isolated secretion cells. a, starch grains; c, cambial cells; cr, crystals of calcium oxalate; m.r., medullary ray; ph, phloem; s.c., secretion cell; s.p., sieve plate; s.t., sieve tube.

* R, T and L indicate the measurements made in the radial, tangential and longitudinal directions respectively; the use of these symbols is suggested by Moll and Janssonius in their "Botanical Pen Portraits 1923."

to 56μ and $T = 49$ to 56 to 70μ . Each of these bands is followed by a band consisting of 1 or 2 layers of broad cells with suberised and lignified walls, measuring approximately $R = 28$ to 42 to 49μ , $L = 18$ to 35 to 56μ and $T = 49$ to 56 to 70μ . The radial walls of the broad cells tend to break, and as a consequence the cork frequently peels off in layers. The broad lignified cork cells stain red with phloroglucin and hydrochloric acid, whereas the narrow ones remain yellowish with the same treatment; all the cork cells are insoluble in 80 per cent. sulphuric acid showing that they are suberised.

The cork is followed by a layer of phellogen cells, which have thin cellulosic walls. The phelloderm sometimes consists of about 12 layers of cells, the layers near the phellogen having smaller rectangular tabular cells regularly arranged, whereas the inner layers have larger irregularly shaped cells which appear to have been displaced due to gliding growth during development. The phelloderm cells have walls which are comparatively thicker and more highly refractive than those of the parenchyma of the phloem; they contain numerous starch grains. A few cells of the phelloderm in some specimens contain yellowish granular contents which stain brown with iodine. The fairly wide band of inner bark (Fig. 2, C) consists of numerous broad medullary rays running radially and alternating with comparatively narrow rays of secondary phloem. The cells of the medullary rays (Fig. 3) have thin cellulosic walls and contain numerous starch grains. The medullary rays are 2 to 4 cells wide, the cells being rounded rectangular or ovoid as seen in a transverse section and have small intercellular spaces. The cells measure $R = 18$ to 28 to 49μ , $L = 32$ to 63 to 123μ and $T = 35$ to 63 to 70μ . The path of the ray becomes irregular and indistinct as it approaches the phelloderm. Occasional cells are partly or completely filled with yellowish granular contents which stain brown with iodine or sudan III.

The sieve tissue (Fig. 3), consisting of sieve tubes, companion cells, and phloem parenchyma, lies closely packed in the narrow rays of the phloem between the broad medullary rays, as seen in transverse sections. The majority of the cells of the phloem parenchyma contain starch grains, a few cells contain crystals of calcium oxalate. In a tangential longitudinal section of the bark, the sieve tissue forms irregular wavy lines, the interspaces being filled with the cells of the medullary rays as shown in Figure 3, B. The numerous cells containing calcium oxalate crystals are best seen in this section; some of the crystals are well-formed prisms with which are usually associated numerous irregularly shaped angular crystals of different sizes forming groups or clusters. The clusters or groups usually measure about 7 to 11 to 18μ and the well-formed prisms 11 to 14μ .

The simple starch grains of the inner bark are smaller than those of the wood; they measure 4 to 10 to 20μ and are rounded or ovoid in form with a central hilum. Occasional grains are 2- to 3- occasionally 4-compound. Mounted in lactophenol and observed under polarised light they show a well-defined maltese cross.

The cambiform tissue (Fig. 3, C) consists of 2 or 3 layers of well-defined rectangular cells forming a complete ring between the bark and the wood.

Most of the roots exhibit a tetrarch primary xylem (Fig. 4, A), but occasional roots are triarch; the primary xylem groups form narrow

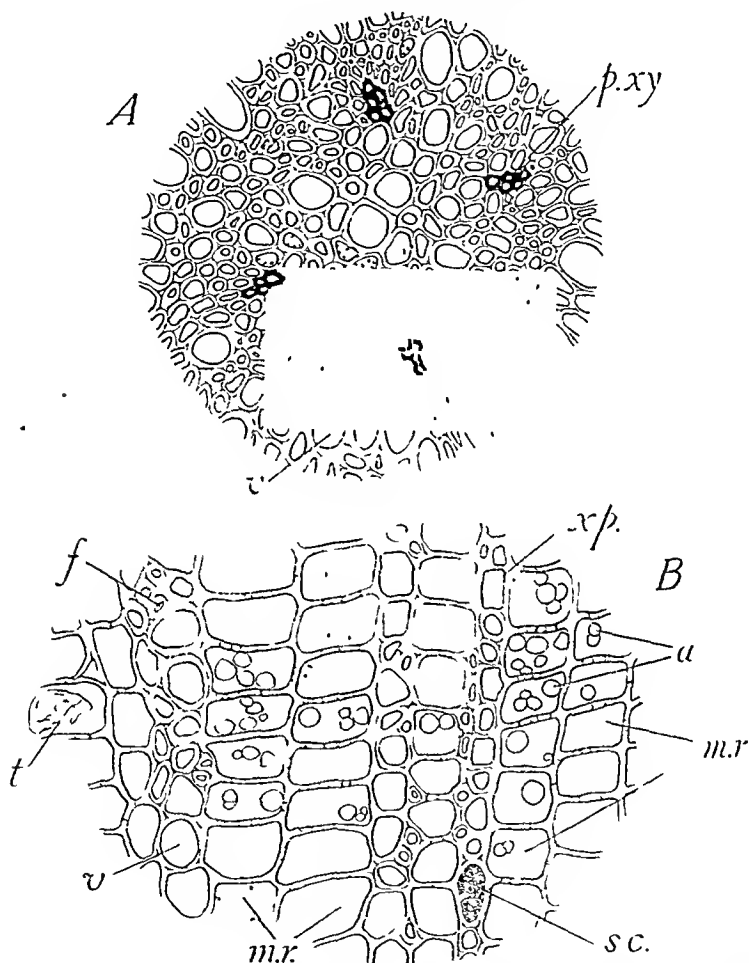


FIG. 4.—*Rauwolfia serpentina* Benth. Root. A, central core of the wood showing primary xylem bundles $\times 200$. B, transverse section of the secondary wood $\times 200$. a, starch grains; f, xylem fibres; m.r., medullary ray; p.xy., primary xylem bundle; s.c., secretion cell; t, resin-like mass; v, vessel; x.p., xylem parenchyma.

triangles as seen in a transverse section. The wood consists of strongly lignified cells (Fig. 2, C), the secondary xylem being arranged in growth rings of varying width, most commercial specimens showing about 3 to 6 seasons' growth. All the cells of the xylem parenchyma (Fig. 4, B) and of the medullary rays contain abundant starch, the grains being simple or 2- to 4-compound; the simple grains measure about 6 to 20 to 46 μ .

the components of the compound grains being rather smaller. Occasional cells of the xylem parenchyma are either completely or partly filled with a yellowish granular substance which stains brownish with iodine or sudan III. In a transverse section the medullary rays are well marked, straight, and continuous with the medullary rays of the phloem. The narrow rays of xylem form anastomosing longitudinally undulating bands. The medullary rays are 1 to 5 cells wide alternating with narrow rays of secondary xylem which consists of vessels, fibres and xylem parenchyma. The longest medullary rays start from a point opposite the apices of the protoxylem groups, others originate at points nearer the circumference making the rays more numerous in the outer growth rings. The cells of the medullary rays measure approximately $R = 14$ to 63 to 86μ , $L = 17$ to 42 to 70μ and $T = 14$ to 42 to 60μ , their walls as well as those of the xylem parenchyma having simple pits except when they adjoin a vessel when they bear half-bordered pits.

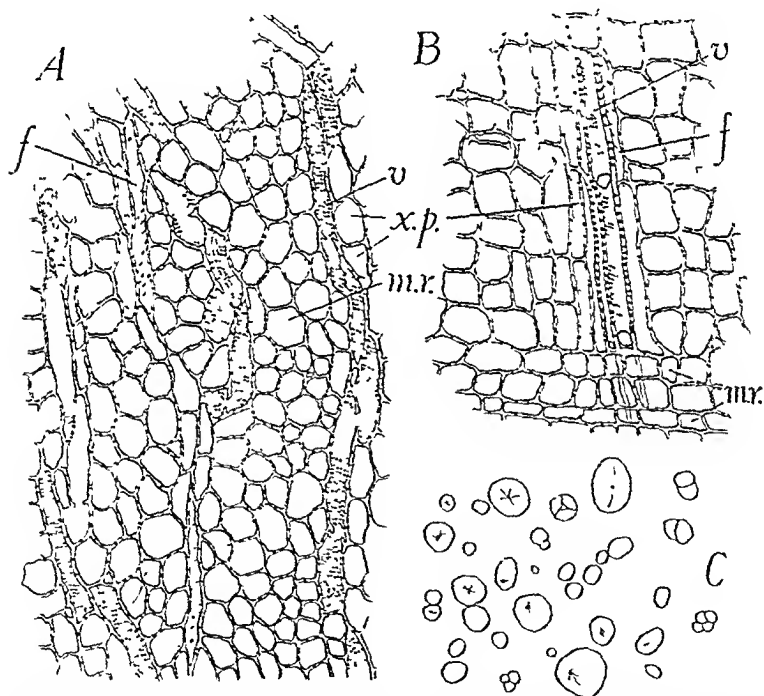


FIG. 5.—*Rauwolfia serpentina* Benth. Root. A, tangential longitudinal section of the wood $\times 100$. B, radial longitudinal section of the wood $\times 100$. C, simple and compound starch grains from powdered drug $\times 200$. f, xylem fibre; m.r., medullary ray, v.p., xylem parenchyma; v, vessel.

The cells of the scanty xylem parenchyma measure about $R = 14$ to 46 to 70μ , $L = 46$ to 53 to 77μ and $T = 14$ to 42 to 56μ .

The vessel elements (Fig. 6), which bear numerous bordered pits, measure about 180 to 234 to 432μ in length and 36 to 54μ in diameter. The transverse or oblique articulations of each element form two rounded

or oval openings either at opposite ends or on the side walls; in the latter case the openings are usually diagonally opposite to each other. The planes of junction of the vessel elements are at right angles to the tangential plane and often lie in the radial plane as shown in Figure 5. Many vessel elements are prolonged at their ends into fibre-like projections. The vessels are associated with numerous xylem fibres having strongly thickened walls and spirally arranged slit-like simple pits. The fibres measure approximately 432 to 576 to 774 μ in length and 18 to

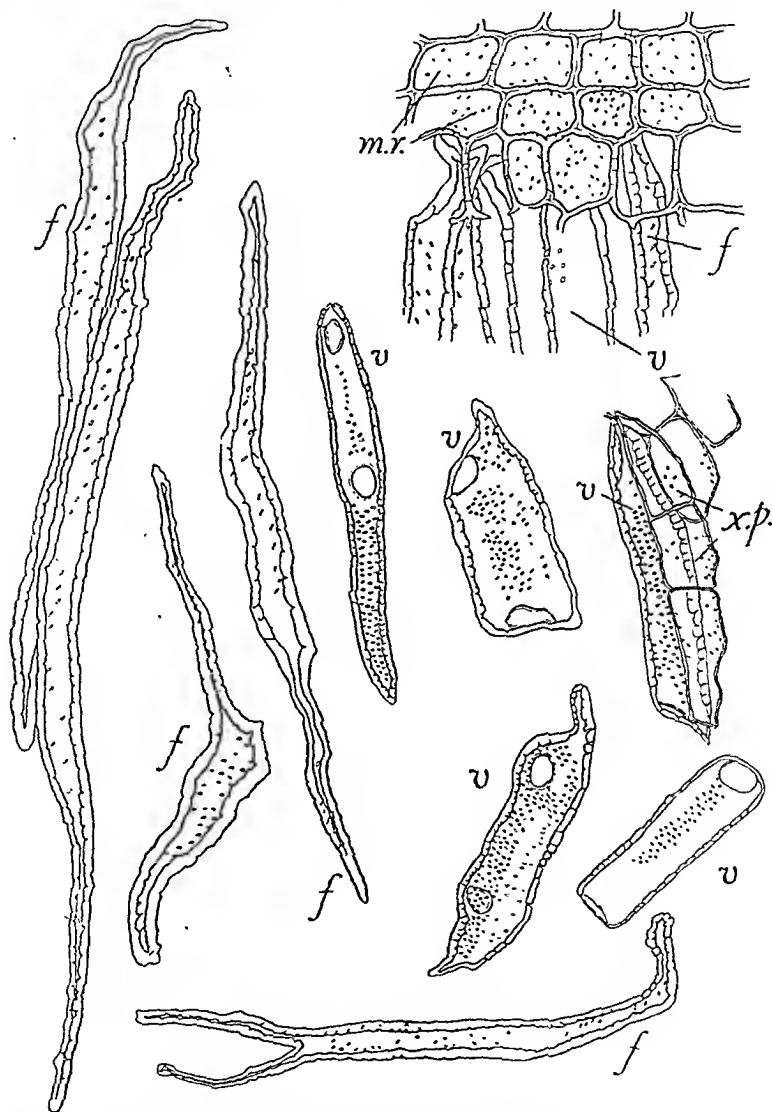


FIG. 6.—*Rauwolfia serpentina* Benth. Root. Isolated elements of the wood from chromic-nitric acid preparation $\times 200$. *f*, xylem fibre; *m.r.*, medullary ray; *x.p.*, xylem parenchyma; *v*, vessel.

36 μ in diameter. Many fibres possess one, or sometimes two, long tapering and often contorted ends. There are very small intercellular spaces between the elements of the xylem.

STRUCTURE OF THE RHIZOME

Histologically, the rhizome differs from the root in certain respects only. The cork of the rhizome (Fig. 7, B) closely resembles that of

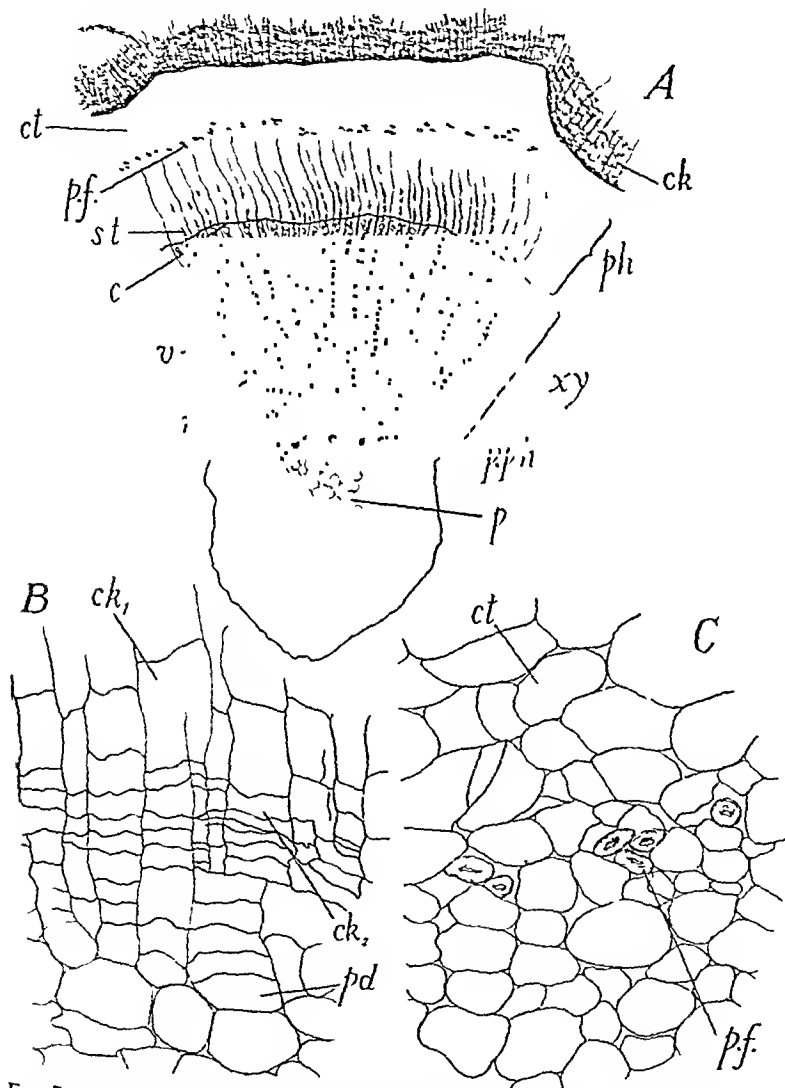


FIG 7.—*Rauwolfia serpentina* Benth. Rhizome. A, diagrammatic transverse section $\times 25$. B, transverse section of the tegumentary tissues $\times 200$. C, transverse section of the pericyclic region; all drawings $\times 200$. c, cambium; ck, cork; ck₁, wide cork cells; ck₂, narrow cork cells; ct, cortex; p, pith; pd, phelloderm; ph, phloem; pph, peri-medullary phloem; p.f., pericyclic fibres; r, growth ring; s.t., sieve tissue; vv, xylem

the root but, owing to the presence of some cells intermediate in radial width between those of the wide and the narrow cells, the layering is less distinct. The phelloderm in the rhizome is narrow and consists of about 3 to 6 layers only, as compared to about 12 layers present in

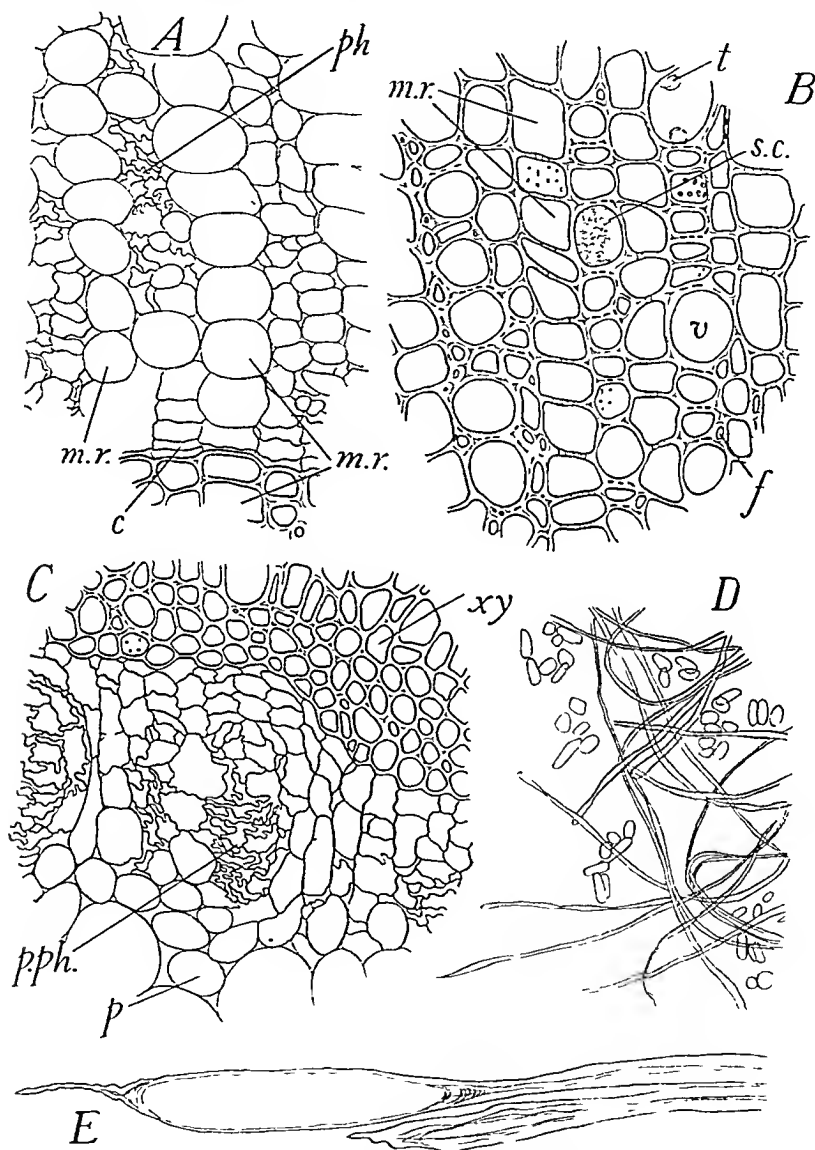


FIG. 8.—*Rauwolfia serpentina* Benth. Rhizome. A, transverse section of phloem adjacent to the cambial tissue $\times 200$. B, transverse section of the wood $\times 200$. C, peri-medullary phloem and pith $\times 200$. D, pericyclic fibres and cells from potash preparation $\times 40$. E, tip of pericyclic fibre showing local enlargement $\times 200$. c, cambium cells; f, xylem fibre; m.r., medullary ray; p, pith; ph, phloem; p.ph., peri-medullary phloem; s.c., secretion cell; t, tylose; v, vessel; xy, xylem.

the root. This is followed by a wide cortex and a pericycle (Fig. 7, C). the cell walls of which are very refractive and in which are present pericyclic fibres, either solitary or in groups of 2 to 4. The fibres (Figs. 8, D and E) have very thick walls and a correspondingly narrow lumen. Many of these fibres show a few scattered elongated ovoid enlargements especially near the ends, a feature which is typical of the Apocynaceæ. These fibres measure about 1.5 mm in length and 7 to 20 μ in diameter with local enlargements about 50 μ in width. Secretion cells are more numerous in the rhizome than in the root and are found chiefly in the cortex and in medullary rays of the phloem. The medullary rays of the phloem (Fig. 8, A) are 1 to 3 cells wide, and the cells measure R = 18 to 28 to 49 μ , L = 46 to 63 to 125 μ and T = 18 to 35 to 77 μ . The starch grains in the rhizome are smaller than those in the root, measuring 2 to 11 to 21 μ . The medullary rays of the xylem (Fig. 8, B) are 1 to 4 cells wide, and the cells measure R = 7 to 32 to 53 μ , L = 14 to 46 to 92 μ and T = 7 to 21 to 49 μ ; the xylem parenchyma is more abundant than in the root (Fig. 8). The vessels closely resemble those of the root, measuring 105 to 308 to 490 μ in length and 25 to 46 to 60 μ in diameter. A peculiar feature of the rhizome, particularly in the older pieces, is the presence, in many of the vessels, of numerous tyloses

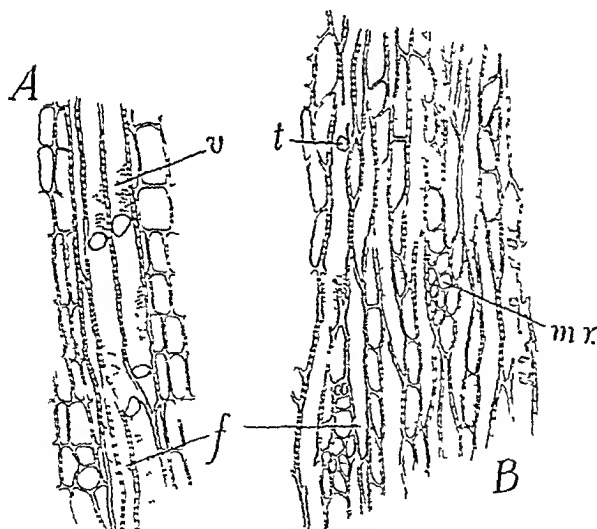


FIG 9—*Rauwolfia serpentina* Benth. Rhizome. A radial longitudinal section of the wood. B, tangential longitudinal section of the wood both drawings $\times 100$ f, xylem fibre, m r, medullary ray, t, tylose, v, vessel

(Figs. 8, B and 9, B) of varying sizes which sometimes almost block the lumen. In some of the vessels exhibiting tylosis, there are granular masses which stain bright red with phloroglucin and hydrochloric acid. Xylem fibres are comparatively abundant in the rhizome and are almost straight; they measure 193 to 560 to 753 μ in length and 11 to 21 to 35 μ in diameter.

The central pith (Figs. 7, A and 8, C) is small, about 0.75 to 1.5 mm. in diameter, and contains in its periphery about 20 small bundles of perimedullary phloem; the remainder of the pith consists of cellulosic parenchyma, the cells of which vary much in size and some of them are filled with a yellowish secretion similar to that in the cortex and phloem.

LATICIFEROUS TISSUE

As the presence of laticiferous tubes is usually regarded as an important characteristic of the Apocynaceæ, a careful search was made for these. No laticiferous tubes were found in the root, but occasional parenchymatous cells in the phloem were found to be filled with yellowish granular contents which stained brown with iodine (Fig. 3, D).

Examination of the rhizome also showed an absence of typical laticiferous tubes: there were, however, a number of secretion cells occurring in the cortex, in the phloem and in the pith, their granular contents staining yellow with iodine or sudan III. Occasionally the secretion cells are arranged in longitudinal rows of 2 to 4, but the transverse walls remain intact.

POWDERED RAUWOLFIA

The salient features of the powdered drug are:—

1. Very numerous rounded and ovoid starch grains about 4 to 20 to 50 μ in diameter, occasional grains being 2- to 4-compound. The starch is rapidly gelatinised in the cold by the action of 0.9 per cent. aqueous solution of potassium hydroxide, the action being more rapid than the similar action of the same solution on potato starch. Strong hydrochloric acid (sp. gr. 1.18) gelatinises the starch instantaneously in the cold.

2. Much lignified rectangular parenchyma having moderately thickened walls bearing simple pits; most of the cells contain numerous starch grains.

3. Fragments of xylem vessels about 36 to 54 μ in diameter, with bordered pits and, associated with them, fragments of the characteristic xylem fibres.

4. Fragments of yellowish cork composed of polygonal-tabular cells of two types, broad and narrow.

5. Small quantities of cellulosic parenchyma, usually filled with starch grains, and occasional unlignified pericyclic fibres showing local swellings.

6. Occasional rounded rectangular secretion cells with yellowish granular contents; and scattered prismatic or irregularly triangular crystals of calcium oxalate.

SUMMARY

1. Rauwolfia of Indian commerce consists of the dried rhizomes and roots, with occasional small pieces of attached aerial stem, of *Rauwolfia serpentina* Benth.

2. The important histological features are the cork, composed of alternating layers of broad and narrow cells, giving a somewhat spongy

PHARMACOGNOSY OF RAUWOLFIA

and friable exterior to the drug; the narrow bark and the wide central mass of wood in the root or broad ring in the rhizome, showing about 3 to 8 growth rings; a tetrarch, or occasionally triarch, primary xylem in roots or a very small pith at the centre of rhizomes; in the periphery of the pith a ring of about 20 small groups of perimedullary phloem.

3. All the cells of the wood, including those of the medullary rays are lignified. The xylem vessels, fibres and parenchyma form narrow undulating radially arranged bands, separated by large medullary rays in the root and by smaller ones in the rhizome. The vessel segments are small and narrow and the xylem fibres are often irregularly shaped with long, usually much contorted, tapering ends; many of the vessels, especially in the rhizome, show tylosis and occasionally contain resinous masses. The phloem also occurs in undulating bands similar to those of the xylem; phloem fibres are absent.

4. In the rhizome, unlignified pericyclic fibres occur, either singly or in groups of 2 to 4; they show the local and sub-terminal elongated-oval enlargements characteristic of the Apocynaceæ.

5. All the parenchymatous tissues, except the cork, contain numerous starch grains, about 4 to 20 to 50 μ in diameter. Cells containing a brownish yellow secretion occur in small numbers, either singly or in short longitudinal rows, in the bark, wood and pith, being most frequent in the cortex of pieces of rhizome. Typical laticiferous tubes are absent. Calcium oxalate occurs in numerous cells of the phloem and of the medullary rays of the bark; the crystals are either well-formed prisms or aggregates of irregular angular components.

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THE STRUCTURE OF γ -SUGARS

PART VI

THE SYNTHESIS OF A FURANOSE, A 6-METHYLKETOHEXOSE

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THE γ -sugars are unstable and have not yet been isolated in the free state. They occur in nature only in glycosidal combination, e.g., in sucrose, inulin, graminin and in sinistrin, on hydrolysis of which only the normal form of the sugar is isolated. Derivatives of many of the hexoses and pentoses are known to exist in the γ -form. The simplest derivatives of these labile sugars are the glycosides, which have been found to differ widely in their chemical properties from those of the normal sugars. Thus they are susceptible to hydrolysis by very dilute mineral acid and can be distilled without decomposition *in vacuo*. Furthermore, the γ -glycosides reduce permanganate solution in the cold, a property which has not yet been shown by any of the glycosides of normal sugars.

Haworth and collaborators are responsible for the accepted explanation of the difference in constitution, the γ -sugars being derived from furan, whilst the normal sugars are similarly related to pyran. In this connection it is of interest to note that Hersant and Linnell¹ obtained by synthesis a methylated hexose which could not possess a five-membered ring and which exhibited properties in agreement with those of the normal sugars. Fischer² first isolated γ -methyl-glucoside and Haworth and Law³ established their occurrence in natural products by proving that the fructose residue in sucrose was a γ -form.

Evidence for the furanose structure of γ -fructose rests mainly on the degradation of tetramethyl- γ -fructose obtained on hydrolysis of heptamethylsucrose through trimethylfructofuronic acid to trimethyl- γ -arabinolactone and then to dimethoxysuccinic acid.^{4,5,6} Other structures^{7,8} have been suggested, but most of them are based upon studies of the oxidation products of derivatives and these oxidations are far from being quantitative. Hartley and Linnell⁹, however, on the basis of parachor studies of partially and fully methylated derivatives of γ -fructose, from the kinetic studies of the hydrolysis of sucrose and on account of the absence of mutarotation in both 3:4:6 trimethyl- γ -fructose and 6-methylfructose solutions, suggested for γ -fructose and its non-glycosidic derivatives an open chain keto-alcohol structure.

The determined parachor values of tetramethyl- γ -fructose and of tetramethyl- γ -methylfructoside were found to be significantly lower than those calculated for the structure assigned to γ -fructose by either Haworth¹⁰ as a five-membered ring or by Hudson¹¹ as a four-membered ring structure. Again five- and six-membered oxygen ring compounds are not normally different in their stability (Linnell and Melhuish¹²).

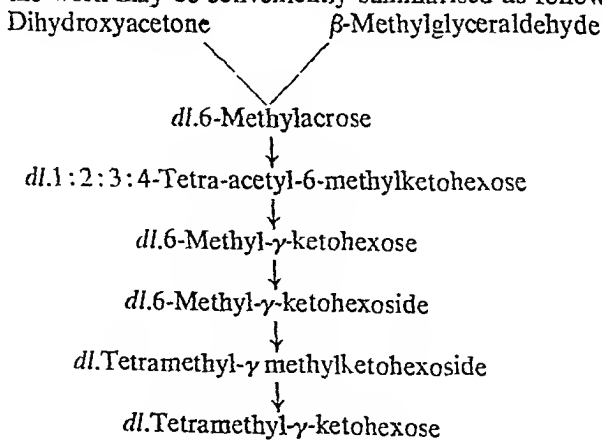
The stability of γ -fructose and the heat of activation of its conversion to normal fructose as obtained from the kinetic studies of sucrose lead to results which are not completely explained by the accepted furanose structure.

The half-life period of γ -fructose as has been shown by Hartley and Linnell¹³ and others^{14,15} to be too small to permit its isolation, and hence any structural enquiries concerning such compounds must rest on a study of their derivatives. The justification for such approach rests on a precise knowledge of the constitution of the derivative used. No derivative of a γ -sugar has yet been synthesised by an unambiguous route, although Hartley and Linnell¹¹ synthesised γ -fructose derivatives which could not exist in pyranose form¹⁶.

It was, therefore, decided to attempt the synthesis of a monomethyl-ketohexose in which the position of the methoxy group would be established beyond doubt by the method used. Such a method would not only avoid the possibility of a ring change during methylation, but would also be free from any criticism which might be directed towards the drastic reactions involved in the oxidative degradation methods adopted by Haworth and his collaborators.

By a modification of Fischer's synthesis of acrose^{17,18}, using pure dihydroxyacetone and monomeric β -methylglyceric aldehyde, a monomethyl-ketohexose was obtained which could not be other than a 6-monomethyl-ketohexose. No 6-monomethyl-ketohexose had been synthesised before.

The preparation of the starting materials for this synthesis have been previously reported¹⁹ and the main series of reactions involved in the remainder of the work may be conveniently summarised as follows:



The condensation of dihydroxyacetone and glyceraldehyde was shown by Fischer¹⁷ to take place in the presence of 1 per cent. of sodium hydroxide. Schmitz²⁰ (see also Jackson²¹ and Neuberg²²) obtained a relatively good yield of acrose using 0.1 per cent. barium hydroxide solution. Hersant and Linnell¹ effected condensation of dihydroxyacetone and methyl- γ -glyceraldehyde by using 0.1 per cent. barium

hydroxide solution for a total period of 5 weeks, sufficient barium hydroxide being added from time to time to maintain the alkalinity of the reaction. In the present research it was decided to employ the latter method as being less likely to induce epimerisation or resinification.

Dihydroxyacetone and β -methylglyceraldehyde in equimolecular proportions were therefore condensed in 5 per cent. aqueous solution in the presence of 0.1 per cent. of barium hydroxide. After a few days the solution became yellow and sufficient barium hydroxide was added from time to time to maintain a well-marked alkalinity, the solution being kept in the dark for a period of six weeks.

Fischer¹⁷ isolated *dl*-fructose from the condensation product through osazone formation, which he converted to osone and then to fructose by reduction. Hersant and Linnell¹ obtained *dl*-5-methylfructose by following the method of Fischer. Isolation was attempted according to Schmitz²⁰, who was able to isolate a crystalline hexose directly. The solution containing the condensation product was therefore neutralised and evaporated to dryness *in vacuo*, the residue being taken up in absolute alcohol, but on evaporation *in vacuo* no crystalline substances could be isolated. Addition of anhydrous ether to the alcoholic solution caused the separation of a white, amorphous substance which gave aggregates of needle crystals (m.pt. 99° to 101°C.) on treatment with phenylhydrazine acetate.

The number of products obtainable in this condensation must include derivations of *dl*-fructose, *dl*-sorbose, *dl*-tagatose and *dl*-psicose, but previous experience has shown¹⁷ that *dl*-fructose predominates, the only other sugar isolated in small quantity being *dl*-sorbose. Since dihydroxyacetone in the alkaline medium may enolise, it is not impossible that derivatives of the eight *dl*-aldoses might be present in the product.

It is not surprising, therefore, that little success crowned the efforts to isolate the osazones directly from the alcoholic solution of the product. A small yield of a crystalline product melting at 130° to 132°C. was obtained, however, and this could be *dl*-methylsorbosazone, but the yield was very small. Recourse was, therefore, made to a different method of isolation through the acetylated product. The acetylation was attempted according to the method of Erwig and Koenigs²³, but the yield was too small. Modification of the process of Hudson and Braun²⁴ (by using dry material, increasing the amount of acetic anhydride, stirring for a longer time, and special treatment of the chloroform emulsion obtained on extraction) gave more promise. After removal of the chloroform the main product was a light yellow syrup together with a few crystals. Keeping over potassium hydroxide *in vacuo* for 14 days caused no alteration. The crystals were probably traces of acetylated aldoses and the syrup—the main product—represented a 69 per cent. yield and gave analytical figures for a tetra-acetylmethylhexose. Saponification indicated that the purity was about 99 per cent. This saponification required special care because the methylhexose resulting may be readily attacked by acids or alkalis. Erwig and Koenigs found that the

liberated fructose was slightly attacked by 3 hours' boiling with 0.1N sulphuric acid.

Hudson and Braun observed a similar action of acid on the liberated fructose and after various modifications discarded the method of acid hydrolysis. They found that if 0.1N sodium hydroxide was used for saponification of fructose penta-acetate by shaking at 0°C., the liberated fructose was not attacked by the amount of alkali present.

Determination of the saponification value of the tetra-acetylmethylhexose was then carried out by shaking with 0.1N sodium hydroxide at room temperature for 4½ hours and followed by titration with 0.1N sulphuric acid using phenolphthalein as indicator. After saponification concentration of the liquid *in vacuo* after neutralisation left a brown syrup which reduced Fehling's solution slowly in the cold, vigorously on warming and reduced alkaline permanganate solution in the cold.

Saponification of larger quantities of tetra-acetylmethylhexose was carried out under the same conditions and the liquid was neutralised with dilute acetic acid, filtered and evaporated to dryness *in vacuo*. The residue was then extracted with absolute alcohol, filtered from the sodium acetate, decolorised with charcoal and concentrated *in vacuo*. It was redissolved in alcohol and reconcentrated until sodium acetate was completely eliminated. A pale yellow syrup remained, which after keeping in a vacuum desiccator for a long time became a toffee-like mass. Methoxyl determination of the product gave 4 figures which were slightly high probably owing to the presence of a little 1:6-dimethylhexose formed by condensation of β -methylglyceraldehyde with monomethyldihydroxyacetone formed by the action of alkali on β -methylglyceraldehyde.

- | | |
|---|--|
| 1. Molisch's test:— | Positive. |
| 2. Barfoed's reagent:— | Reduction on warming. |
| 3. Fehling's solution:— | Reduction slowly in the cold, vigorous on warming. |
| 4. Ammoniacal silver nitrate solution:— | Reduction in the cold, mirror on warming. |
| 5. Schiff's reagent:— | Magenta colour slowly. |
| 6. Alkaline potassium permanganate solution:— | Instantaneous reduction in the cold. |
| 7. Methyl alcoholic hydrochloric acid cold for 1 hour:— | Syrup did not reduce Fehling's solution, but reduced permanganate in the cold. |
| 8. Phenylhydrazine acetate solution:— | Needle crystalline osazone m.pt. 130° to 132°C. |
| 9. After standing in a vacuum desiccator for 3 months, 6-methylketo-hexose was found unchanged and still reduced alkaline potassium permanganate in the cold. | |
- Reduction of Fehling's solution and potassium permanganate in the cold, reaction with Schiff's reagent and formation of a methylglycoside

in the cold are held to be characteristics of γ -sugars and are not given by normal sugars under the specified conditions.

Glycoside formation was accomplished by dissolving a quantity of *dl*- γ -methylhexose in methyl alcohol containing 1 per cent. of dry hydrogen chloride and keeping the solution at room temperature for 2 hours. On isolation a yellow syrup remained which failed to reduce Fehling's solution even after warming, but reduced alkaline permanganate in the cold. Any glycoside obtained by this established method should be a γ -glycoside; its behaviour towards permanganate together with its syrupy consistency differentiates it from the crystalline normal glycopyranosides.

Further indication of the nature of this glycoside was given on attempting its hydrolysis with N/100 aqueous hydrochloric acid: the hydrolysis was complete in 1 hour, whereas γ -methylglucopyranoside was hardly affected under these conditions.

The mixture of 6-methylhexoses was methylated with dimethylsulphate and sodium hydroxide²⁵ followed by Purdie's²⁶ reagent. The product on isolation did not crystallise and gave analytical figures for a tetramethyl-methylhexoside. It did not reduce Fehling's solution, but reduced permanganate in the cold. On hydrolysis it gave a liquid product which reduced Fehling's solution and potassium permanganate in the cold.

The product of the condensation of β -methoxy- α -hydroxypropionaldehyde and dihydroxyacetone possessed properties which sharply differentiated it from a pyranose. The condensation could not yield an amylenoxide structure owing to the blocking of the 6-hydroxyl group. It could possess a ketone structure or a 1:2, 2:3, 2:4 and 2:5-oxide rings of which the 2:5 is by far the most likely. The results of this synthetic experiment indicates that hexoses possessing other than the amylenoxide structure exhibit properties far more reactive than the normal sugars. These differences are greater than would be expected between derivatives of furane and pyrane, but the results of these experiments are difficult to explain on any other basis.

The separation of the product of this synthesis into individual sugars by partition of the acetylated material by chromatographic methods will be communicated later.

EXPERIMENTAL

Condensation of β -methoxy- α -hydroxypropionaldehyde and dihydroxyacetone.—Freshly prepared β -methoxy- α -hydroxypropionaldehyde (20 g.) dissolved in 400 ml. of water was mixed with a solution of dihydroxyacetone (20 g.) in 400 ml. of water and 1 g. of barium hydroxide dissolved in a little hot water was added to the mixture. The solution was kept protected from light for 6 weeks, sufficient barium hydroxide being added from time to time to maintain a well-marked alkalinity. The solution became yellow in colour during the condensation.

Isolation of the products.—(a) An attempt to isolate by treatment of the above solution with phenylhydrazine-acetate mixture resulted in the

formation of a black oil from which a small quantity of pale yellow needles melting at 130° to 132°C. was obtained.

(b) The condensate was neutralised with dilute sulphuric acid and the filtered solution was evaporated *in vacuo* at 30° to 35°C. The light brown syrupy residue was dissolved in absolute alcohol and filtered. On removal of the alcohol no crystals appeared and the syrupy residue was redissolved in alcohol and diluted with ether when a voluminous, white, amorphous precipitate formed. After separation, washing with ether and drying over sulphuric acid a hygroscopic creamy product was obtained which reduced Fehling's solution on warming and ammoniacal silver nitrate solution in the cold.

The alcohol-ether filtrate was evaporated at ordinary temperature giving a light brown syrup which reduced Fehling's solution and ammoniacal silver nitrate solution.

6-Methylketohehexose.—The solution (800 ml.) containing the condensation products was carefully neutralised and the clear filtrate was evaporated under reduced pressure at 30° to 35°C. , a little alcohol being added towards the end of the process. A viscous yellow syrup (35 g.) was obtained which gave all the tests characteristic of a γ -sugar. In all some 200 g. was prepared.

6-Methyltetra-acetylketohehexose.—The dried 6-methylketohehexose (25 g.) was slowly added to a mixture of 150 ml. of acetic anhydride and 6.25 ml. of sulphuric acid, the mixture being strongly cooled. On stirring in the cold for about 2 hours complete solution was effected. The mixture was poured into 600 ml. of ice-cold water, neutralised with sodium bicarbonate, filtered and the residue, consisting of the acetylated sugar, was dissolved in chloroform. The filtrate was repeatedly extracted with chloroform, the chloroform solutions were washed with water, dried, treated with activated charcoal, filtered and evaporated under reduced pressure. The syrupy residue was kept over potassium hydroxide *in vacuo*, when signs of crystallisation appeared but the bulk of the material remained as a yellow syrupy liquid. Yield 32 g. (69 per cent.) Found C, 49.07; H, 7.03 per cent.; $\text{C}_{15}\text{H}_{22}\text{O}_{10}$ requires C, 49.72; H, 7.08 per cent. 0.5086 g. of syrup required 55.70 ml. of N/10 sodium hydroxide for complete saponification; theory requires 56.10 ml. of N/10 sodium hydroxide.

Purified 6-methylketohehexose.—The tetra-acetate (75 g.) was hydrolysed with N/10 sodium hydroxide and after isolation in the usual way a yellow, very viscous syrup was obtained which reduced Fehling's solution slowly in the cold but vigorously on warming, reduced potassium permanganate solution in the cold and formed a glycoside on treatment with methyl alcoholic hydrogen chloride at normal temperature for 1 hour. These properties are characteristic of a γ -sugar. A determination of methoxyl using a modified Pregl method gave OCH_3 , 16.78 per cent.: $\text{C}_9\text{H}_{14}\text{O}_5(\text{OCH}_3)$ requires 15.97 per cent. The slightly high figure was probably due to the presence of a small quantity of dimethyl derivative (*vide supra*).

6-Methyl- γ -methylketohexoside.—A solution of 6-methylketohexose (1 g.) in 15 ml. of pure methyl alcohol containing 1 per cent. of dry hydrogen chloride was kept at room temperature for 2 hours. The solution was neutralised with silver carbonate, filtered and the solvent removed by distillation. The residue, a brown syrup, failed to reduce Fehling's solution even after warming, but it reduced potassium permanganate solution in the cold. On hydrolysis the power to reduce Fehling's solution was restored.

Hydrolysis of 6-methyl- γ -methylhexoside.—A solution of 6-methyl- γ -methylhexoside (0.69 g.) in 12 ml. of N/100 hydrochloric acid was placed in a water-bath at 95°C. At intervals 0.2 ml. of the solution was removed and added to 1 ml. of Fehling's solution diluted with 2 ml. of water. The solution was replaced in the water-bath and the amount of reduction was observed at the end of 5 minutes. The results were compared with those of methylglucopyranoside treated similarly.

Time in Minutes after Beginning Heating at 95°C.	Reduction Observed	
	6-methyl- γ -methyl-ketohexoside	methylglucopyranoside
5	Distinct reduction	Very slight reduction
10		
15	Strong reduction	Slight reduction
30	Rapid	" "
60	Complete "	" "

Tetramethyl- γ -methylketohexoside.—The 6-methylketohexose was methylated with dimethyl sulphate and sodium hydroxide by the normal method, at first at 30°C. and then at 70°C. After isolation the process was repeated and the product was worked up in the usual manner. The product was then further methylated, using methyl iodide and silver oxide. On isolation 12 g. of a yellow syrup was obtained which showed no reduction of Fehling's solution, but still reduced potassium permanganate solution in the cold. Found OCH_3 , 60.19 per cent.; $\text{C}_6\text{H}_7\text{O}(\text{OCH}_3)_5$ requires OCH_3 , 62 per cent.

Tetramethyl- γ -ketohexose.—Hydrolysis of the tetramethyl- γ -methylketohexoside (1 g.) was effected with 1 per cent. aqueous hydrochloric acid by heating under reflux for half an hour. After neutralisation and isolation in the usual manner a syrup remained which reduced Fehling's solution and potassium permanganate solution in the cold.

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A CHLOROHYDROXY-TRIPHENYLMETHANE DYE

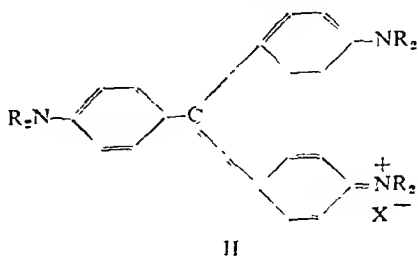
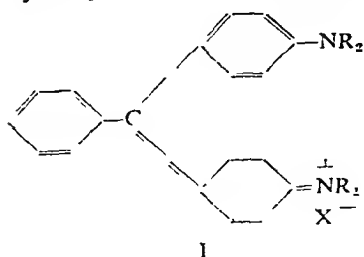
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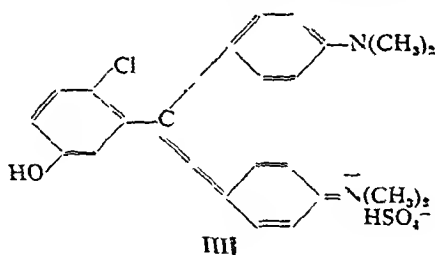
EHRlich and Bechhold¹ first showed conclusively that the introduction of a halogen atom into the aromatic nucleus produced an increase in the bactericidal activity of phenols. Karpow² had already demonstrated that of the three isomeric chlorophenols the para-compound was the most active. These results received further confirmation in later work carried out by Kurodo³ although Klarmann⁴ showed that the activities of the three chlorophenols increased in the order *ortho*-, *para*-, *meta*-. An increase in the molecular weight of both simple phenols and halogenated phenols by the introduction of alkyl, aryl and aryl-alkyl groups into the molecule causes a marked increase in activity. Klarmann, Schternov and Gates⁵ tested a series of alkylchlorophenols against 6 different micro-organisms and showed that whereas a peak of activity against *Eberthella typhi* was obtained in those compounds with 5 carbon side chains the peak of activity against *Staphylococcus aureus* and *Streptococcus haemolyticus* was only attained when the side chain contained 7 or 8 carbon atoms. Furthermore *o*-alkyl-*p*-chlorophenols are more active than *o*-chloro-*p*-alkylphenols. In a corresponding series of benzylhalophenols Klarmann, Gates and Schternov⁶ showed that a halogen which is *ortho* to a hydroxyl group increases the phenol coefficient less than when in the *para*-position, though the general level of activity in these diphenylmethane derivatives is lower than that for the corresponding alkylhalophenols. 5-Chloro-2-hydroxydiphenylmethane has approximately half the activity of 2-*n*-amyl-4-chlorophenol toward *Staph. aureus* and *Strep. haemolyticus*. Contrary to the greater activity against both Gram-negative and Gram-positive organisms of bromophenols over chlorophenols in the simpler series, the monobromo derivatives of 2- and 4- hydroxydiphenylmethanes are less effective against Gram-negative organisms yet more so against Gram-positive types than the corresponding chloro compounds.

Basic dyes of the triphenylmethane series such as malachite green (I, R=CH₃, X=Cl), brilliant green (I, R=C₂H₅, X=HSO₄) and crystal violet (II, R=CH₃, X=Cl) are well established as antibacterial agents, yet little attention has been paid to the possibilities of their chloro- and hydroxy- derivatives as potential bactericides. New solid green 3B.



which is 2-chloro-4':4''-bisdimethylaminotriphenylmethyl chloride was found by Beckwith⁷ to be active against typhoid in rabbits. No comparison of the activity of this compound with that of malachite green is recorded. Kligler⁸ compared the activity of victoria green, 2:5-dichloro-4':4''-bisdimethylaminotriphenylmethyl chloride with that of malachite green, and showed that the former was slightly more active than the latter. The effect of introducing an hydroxy-group into the molecule of malachite green structure was studied by Fairbrother and Renshaw⁹. 3-Hydroxy-4':4''-bisdimethylaminotriphenylmethyl chloride was found to be too insoluble for testing, while the more soluble patent blue V, monosodium - 5 - hydroxy - 4':4''-bisdimethylaminotriphenylmethyl-2:4-disulphonate possessed no antiseptic properties. Simon and Wood¹⁰ had concluded earlier that all predominantly acidic triphenylmethane dyes are inactive and these findings were confirmed by Fairbrother and Renshaw⁹. But, in view of the presence of two sulphonic acid groups in the molecule of patent blue V, no conclusion can be drawn as to the effect of introducing the hydroxy group into the triphenylmethane molecule.

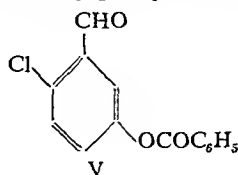
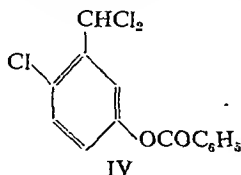
The compound III, 2-chloro-5-hydroxy-4':4''-bisdimethylamino-triphenylmethyl sulphate, embodies the salient properties of two types of antibacterial substances. One of the aromatic rings introduces the



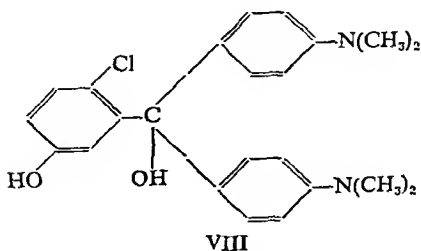
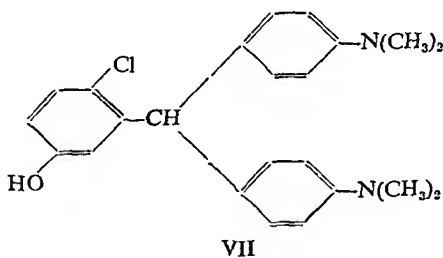
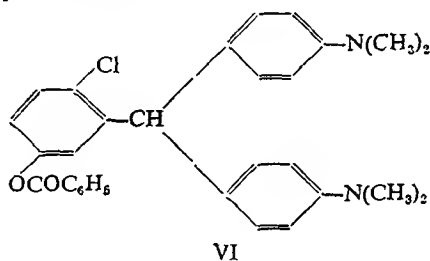
parachlorophenol groupings into the molecule, while an arylalkyl side chain is duplicated in the main triphenylmethane structure. Such a compound might be expected to combine the selective antibacterial action of the triphenylmethane dyes with the more general bacterial toxicity associated with phenolic substances and provide the basis for a new and powerful series of bactericides.

For the synthesis of III, 2-chloro-5-hydroxytoluene was chosen as a convenient starting point on account of its ready availability. The required orientation of chloro- and phenolic groups with respect to the alkyl side chain was thus already established. The oxidation of the alkyl side chain to the aldehyde was accomplished by chlorination to introduce two chlorine atoms, followed by hydrolysis. In the first instance the phenolic group was protected by acetylation and the acetyl derivative chlorinated in bright light at 110°C. to give the theoretical increase in weight for the introduction of two chlorine atoms. On cooling the product solidified and after re-crystallisation was identified as 2:4:6-trichloro-3-acetylcresol, m.pt. 35°C. It seemed possible that side chain chlorination could be achieved with a derivative of higher boiling-point and this expectation was realised with benzoyl-2-chloro-5-hydroxy-

toluene. This compound was chlorinated, in bright light, at an initial temperature of 140°C . which was slowly increased to 180°C . during the course of the reaction. 4-Chloro-3-dichloromethylphenyl benzoate (IV)



was obtained as a pale yellow, viscous oil after fractional distillation *in vacuo*. This substance was easily converted into the corresponding aldehyde, 2-chloro-5-benzoylhydroxy-benzaldehyde (V) using the method of Hammick¹¹, boiling with alcoholic silver nitrate solution for 20 minutes. After concentration V crystallises from the filtrate as a white microcrystalline solid which readily forms a 2:4-dinitrophenylhydrazone and a semicarbazone. Its identity was confirmed by titration using hydroxylamine hydrochloride.



2-Chloro-5-benzoylhydroxy-4':4''-bisdimethylaminotriphenylmethane (VI) was obtained in 70 per cent. yield by condensing the aldehyde (V) with dimethylaniline, using phosphorus oxychloride as the condensing

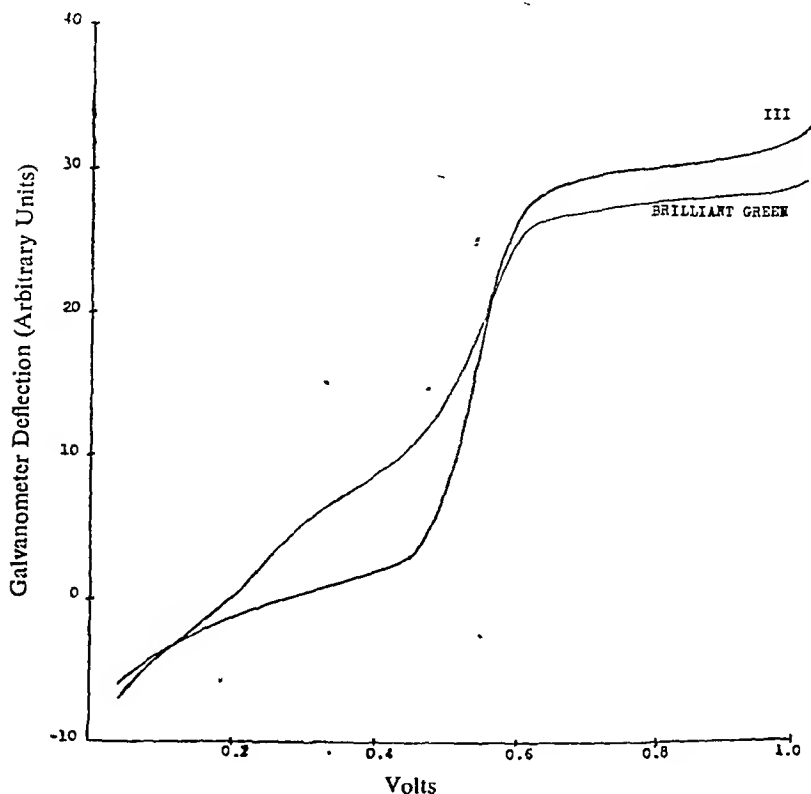
agent and *o*-dichlorobenzene as solvent, according to the conditions of Stryker¹². The product twice recrystallised from alcohol (95 per cent.) was a white crystalline powder and readily formed a picrate which, on analysis and titration with standard sodium hydroxide solution, was shown to possess the formula $B_1[C_6H_2(NO_2)_3OH]_2$.

Debenzoylation of VI to 2-chloro-5-hydroxy-4':4''-bisdimethylamino-triphenylmethane (VII) was achieved by refluxing with alcoholic solution of potassium hydroxide. The crude leucobase (VII) was separated in 98 per cent. yield in the form of dark green crystals. Purification was effected by chromatographic adsorption from benzene on a column of activated alumina, followed by recrystallisation from alcohol (95 per cent.) to give a white crystalline product in 90 per cent. yield. The picrate was obtained as a yellow crystalline solid and was shown by analysis and by titration against standard solution of sodium hydroxide to have the formula $B_1[C_6H_2(NO_2)_3OH]_2$.

Oxidation of VII to the carbinol base, 2-chloro-5-hydroxy-bisdimethylamino-triphenylmethylcarbinol (VIII) was carried out by the method of Minevitch¹³ in 70 per cent. acetic acid at 0° to 5°C., using lead peroxide paste. The carbinol base (VIII) was obtained as a dark green scale product in 89 per cent. yield after purification by chromatographic adsorption on activated alumina from chloroform and elution from the column with the same solvent. It has no distinct melting-point and undergoes slow fusion with decomposition when heated for any length of time at temperatures over 100°C. It is insoluble in water, benzene, light petroleum (b.pt. 50° to 60°C.), ether and carbon tetrachloride; soluble in alcohol and very readily soluble in chloroform and acetone. The picrate of VIII was obtained as a dark yellowish green powder and was shown by analysis to possess the formula $B_1[C_6H_2(NO_2)_3OH]_2$.

The conversion of the carbinol base (VIII) to the dyestuff, 2-chloro-5-hydroxy-4':4''-bisdimethylaminotriphenylmethyl sulphate (III) was accomplished by agitating a chloroform solution of VIII with water containing the calculated amount of sulphuric acid, and evaporating to dryness. The identity of the product was established by microanalysis, though direct titration on a semi-micro scale with titanous sulphate solution using the method defined for brilliant green in the British Pharmacopœia failed to give consistent results. Further confirmation of the identity of III was obtained by a study of its polarographic wave in comparison with that of a pure sample of brilliant green. The waves were plotted using a voltameter on solutions buffered at approximately pH2 with hydrochloric acid and 0.1N potassium chloride, the latter substance serving also as the ground electrolyte. Gelatin at a concentration of 0.02 per cent. was used for "maximum" suppression and alcohol to facilitate solution of the dyestuffs. A blank was carried out to demonstrate the absence of interference of the ancillary substances present in the solution. Two typical waves are shown in Figure I. These are followed closely by a large hydrogen wave. The waves of the dyestuff (III) and of brilliant green are shown to be comparable. The half-wave potentials are

0.535V and 0.53V for III and brilliant green respectively, and are measured with respect to the pool mercury anode. The waves are completely cathodic, which may be taken as evidence of completeness of oxidation to the dye form.



The dye III is only sparingly soluble in water, insoluble in ether, benzene and light petroleum, but soluble in acetone, chloroform and alcohol. It is capable of dyeing both silk and wool directly, and cotton in the presence of suitable mordants. The green colours so obtained remain fast on exposure to ultra-violet light for short periods, but are not fast to the action of boiling soap solutions. Preliminary bacteriological tests in aqueous solutions showed that III inhibited the growth of *Streptococcus pyogenes* at a dilution of 1/80,000 and *Staphylococcus aureus* at more than 1/160,000. A 1/1000 solution did not inhibit the growth of *Escherichia coli* and *Pseudomonas pyocyanea*.

CONCLUSION

The chlorohydroxy-triphenylmethane dye III has been shown to possess a low order of antibacterial activity. The results of Fairbrother and Renshaw indicated that the presence of an acidic group in the molecule of a basic triphenylmethane dye, would decrease the activity. The intro-

duction of the phenolic group into the molecule of the dye III has demonstrated this effect and any enhancement of potency due to the presence of the chloro-group was insufficient to compensate for the reduction in activity due to the phenolic group.

EXPERIMENTAL

3-Methyl-4-chlorophenyl benzoate.—2-chloro-5-hydroxytoluene (71.2 g.) was dissolved in 5N sodium hydroxide solution (100 ml.). Benzoyl chloride (70.2 g.) was added slowly and the mixture shaken continuously for 10 minutes. A further addition of 5N sodium hydroxide solution (10 ml.) was made and the shaking repeated. The solid product was filtered from the solution, washed first with dilute sodium hydroxide solution, then with water, dried and recrystallised from alcohol (95 per cent.). Yield 110.5 g. (90 per cent.), m.pt. 86°C.

3-Dichloromethyl-4-chlorophenyl benzoate (IV).—3-methyl-4-chlorophenyl benzoate (110.5 g.) was heated to 140°C. and a current of dry chlorine was slowly passed into the molten material for 9 hours. During the course of the chlorination the temperature was allowed to rise slowly to 180°C., and the apparatus was exposed to sunlight. The product, a dark brown viscous oil, was distilled *in vacuo*, and the second fraction, b.pt. 203° to 204°C./4 mm. Hg, was collected. Yield 94 g. (67 per cent.). μ . 1.6013. Found: C, 52.90; H, 2.94; Cl, 35.36. $C_{14}H_9O_2Cl_3$ requires C, 53.26; H, 2.88; Cl, 33.76 per cent.

2-Chloro-5-benzoylhydroxybenzaldehyde (V).—Silver nitrate (56 g.) was dissolved in distilled water (60 ml.), heated to 80°C. and added with continuous stirring to a solution of IV (51 g.) in boiling alcohol (95 per cent.) (250 ml.). The whole was refluxed for 20 minutes with continuous and vigorous stirring and then allowed to cool. Neutralisation was effected by the careful addition of the calculated amount of calcium carbonate and the precipitated silver chloride removed by filtration, washed with alcohol (95 per cent.) and dried. The filtrate was evaporated to dryness, the residue extracted with boiling absolute alcohol and filtered. On cooling V separated as a white crystalline solid, m.pt. 94° to 94.5°C. (Corr.). Yield (i) 34.1 g. A further yield of product was obtained by submitting the dried precipitated, silver chloride to continuous extraction with alcohol (95 per cent.), evaporating off the excess of alcohol and allowing to crystallise. Yield (ii) 1.5 g. Finally the combined mother liquors were concentrated and shaken with a saturated solution of sodium bisulphite. The aldehyde-bisulphite compound separated from the solution as a white solid which was filtered, washed with alcohol and then decomposed with sodium carbonate solution. The purified product was extracted with ether, the solution dried with anhydrous sodium sulphate, and the ether removed by evaporation. Yield (iii) 2.1 g. Total yield 37.7 g. (89.5 per cent.). Found: C, 63.19; H, 3.82; Cl, 14.62 per cent. Eq. wt., 263.1. $C_{14}H_9O_3$ requires C, 64.49; H, 3.48; Cl, 13.61 per cent. Eq. wt., 260.5. The aldehyde (V) forms a

2:4-dinitrophenylhydrazone, which crystallises from absolute alcohol in orange prisms, m.pt. 240° to $241^{\circ}\text{C. (Corr.)}$. Found: C, 54.55; H, 3.05; N, 12.80; Cl, 8.76 per cent. $\text{C}_{20}\text{H}_{13}\text{O}_6\text{N}_4\text{Cl}$ requires C, 54.46; H, 2.97; N, 12.71; Cl, 8.05 per cent. The semicarbazone of V is a white microcrystalline solid. It is practically insoluble in alcohol, but may be recrystallised from dry acetone, m.pt. 225° to $226^{\circ}\text{C. (Corr.)}$. Found: C, 56.31; H, 3.27; N, 12.8; Cl, 11.8 per cent. $\text{C}_{15}\text{H}_{12}\text{O}_3\text{N}_3\text{Cl}$ requires C, 56.68; H, 3.81; N, 13.22; Cl, 11.16 per cent.

2-Chloro-5-benzoylhydroxy-4':4''-bisdimethylaminotriphenylmethane (VI)—V (13 g.) was refluxed at 100°C. for 3 hours with dimethylaniline (12.2 g.) and phosphorus oxychloride (7.7 g.), using *o*-dichlorobenzene (23 g.) as solvent. 5N sodium hydroxide solution (30 ml.) and water (30 ml.) were added and the *o*-dichlorobenzene removed by steam distillation. The crude product separated as a dark green sticky mass, which was then extracted with dilute hydrochloric acid (10 per cent.). The extract was filtered, diluted with distilled water (2 l.) and neutralised by the careful addition of strong ammonia solution. A pale green flocculent precipitate was obtained, which was filtered, washed dried and recrystallised from alcohol (95 per cent.), to give a white crystalline solid. Yield 17 g. (70.2 per cent.), m.pt. $150^{\circ}\text{C. (Corr.)}$. Found: C, 71.74; H, 5.90; N, 5.56; Cl, 7.26 per cent. $\text{C}_{30}\text{H}_{29}\text{O}_2\text{N}_2\text{Cl}$ requires C, 74.30; H, 6.02; N, 5.77; Cl, 7.31 per cent. Picrate $\text{B}_1(\text{C}_6\text{H}_3\text{O}_7\text{N}_3)_2$, m.pt. 147.5° to $148^{\circ}\text{C. (Corr.)}$. Found: C, 52.65; H, 3.59; N, 11.50; Cl, 2.98 per cent. Eq. Wt., 970.1. $\text{C}_{42}\text{H}_{33}\text{O}_{16}\text{N}_8\text{Cl}$ requires C, 53.47; H, 3.74; N, 11.86; Cl, 3.76 per cent. Eq. Wt., 942.5.

2-Chloro-5-hydroxy-4':4''-bisdimethylaminotriphenylmethane (VII)—VI (15.7 g.) was refluxed for 4 hours with N/2 alcoholic potassium hydroxide (300 ml.). When cold the solution was just neutralised by the addition of N hydrochloric acid and the alcohol removed by distillation. The solution was made just alkaline by the addition of potassium carbonate, and the solid product which separated was filtered from the solution, washed with water and dried. Yield of crude product 12.13 g. (98 per cent.). This dark green crystalline material was purified by chromatographic analysis, using a 1 per cent. solution in benzene on a column of activated alumina. Development of the chromatogram with a mixture of 10 parts of alcohol (95 per cent.) and 90 parts of benzene caused a separation into four distinct zones, which were coloured (a) dark green, (b) pink, (c) violet, (d) yellow in order from top to bottom of the column. These four fractions were collected separately by continued elution of the column with the developing solvent. Fractions (a), (b) and (c) contained only traces of unidentified impurities. The solvent was removed from fraction (d) under reduced pressure, in a current of hydrogen to minimise oxidation and the pale green solid recrystallised from freshly distilled alcohol (95 per cent.) to give a product which was pure white. Yield 10.91 g., m.pt. 179° to $180^{\circ}\text{C. (Corr.)}$. Found: C, 72.01; H, 6.37; N, 7.62; Cl, 9.84 per cent. $\text{C}_{23}\text{H}_{25}\text{ON}_2\text{Cl}$ requires C, 72.53; H, 6.61; N, 7.35; Cl, 9.31 per cent. Picrate

$B_1(C_6H_3O_2N_3)_2$, m.pt. 187° to $188^\circ C.$ (Corr.), with initial softening at $185^\circ C.$ Found: C, 49.83; H, 3.64; N, 13.5; Cl, 3.83 per cent. Eq. wt. 835.1. $C_{25}H_{31}O_{15}N_5Cl$ requires C, 50.07; H, 3.70; N, 13.36; Cl, 4.23 per cent. Eq. wt. 838.7.

2-Chloro-5-hydroxy-4':4''-bisdimethylaminotriphenylcarbinol (VIII)—VII (5.95 g.) was dissolved in 70 per cent. acetic acid (50 ml.) and the solution cooled to between 0° and $5^\circ C.$ The theoretical quantity of lead peroxide paste (prepared according to Gattermann¹⁴) suspended in 70 per cent. acetic acid (40 ml.) was added slowly to the above solution with continuous stirring over a period of about 10 minutes. The reaction was allowed to proceed at 0° to $5^\circ C.$ for one hour with continuous stirring. Sufficient sodium sulphate solution to precipitate all the lead present was added and stirring continued for 20 minutes. The deep green solution was filtered to remove lead sulphate, made alkaline with sodium bicarbonate, and the precipitated carbinol base extracted with chloroform. The solvent was removed by distillation, and the dark green solid, after drying, continuously extracted with chloroform in a soxhlet apparatus. The chloroform solution was evaporated to 150 ml. and passed through a column of activated alumina. The dark green chromatogram was developed using a mixture of 90 parts of chloroform and 10 parts of alcohol (95 per cent.), when a gradual separation occurred into an upper dark green band and a lower yellow one. The latter was eluted from the column using the same solvent to give a yellow solution, which during the course of evaporation became pale green and finally left a dull green residue (0.15 g.) of unchanged leucobase. The contents of the dark green band on the column were eluted using a mixture of 80 parts of chloroform and 20 parts of alcohol (95 per cent.), and on evaporation of the solvent the product was obtained as a friable, dark, purplish-black solid. Yield 5.39 g. A second fraction (0.10 g.) was obtained by extruding the column, extracting continuously with chloroform for $2\frac{1}{2}$ hours and evaporating the solvent. Total yield 88.6 per cent. Found: C, 68.55; H, 6.42; N, 7.09; Cl, 9.58 per cent. $C_{23}H_{23}O_2N_2Cl$ requires C, 69.56; H, 6.30; N, 7.06; Cl, 8.94 per cent. Picrate $B_1(C_6H_3O_2N_3)_2$. Found: C, 49.65; H, 3.94; N, 13.30; Cl, 4.26 per cent. $C_{35}H_{31}O_{16}N_5Cl$ requires C, 49.12; H, 3.65; N, 13.10; Cl, 4.15 per cent.

2-Chloro-5-hydroxy-4':4''-bisdimethylaminotriphenylmethyl sulphate (III)—VIII (3 g.) was dissolved in chloroform (30 ml.). The calculated volume of N sulphuric acid solution and water (20 ml.) were added with continuous stirring, and the mixture maintained at 45° to $50^\circ C.$ for 1 hour. The dye which is almost insoluble in water separates as a sticky mass which is obtained in fine scales on evaporation of the solvents and drying at $100^\circ C.$ Found: C, 58.92; H, 5.68; N, 5.65; Cl, 7.15; S, 6.79 per cent. $C_{23}H_{23}O_5N_2ClS$ requires C, 57.9; H, 5.68; N, 5.88; Cl, 7.44; S, 6.71 per cent.

Polarography. Polarographs of the dye (III) and of brilliant green were plotted under the same conditions using a voltameter. and these are

illustrated in Figure 1. The composition of the two solutions used was as follows:—

Dye (VI), or brilliant green	5×10^{-4} M.
KCl	10^{-1} M.
Gelatin	0.02 per cent.
0.1N Hydrochloric acid.....	21 ml.
Alcohol (95 per cent.)	50 ml.
Distilled water to	100 ml.

Dissolved oxygen was removed from the solution by passing a stream of hydrogen for 10 minutes, and the polarographs were plotted under the following conditions: temperature 23.5°C., drop time of the mercury cathode 1.4 secs., height of the mercury head 71.5 cm.

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THE SPECTROPHOTOMETRIC DETERMINATION OF RUTIN AND QUERCETIN IN MIXTURES

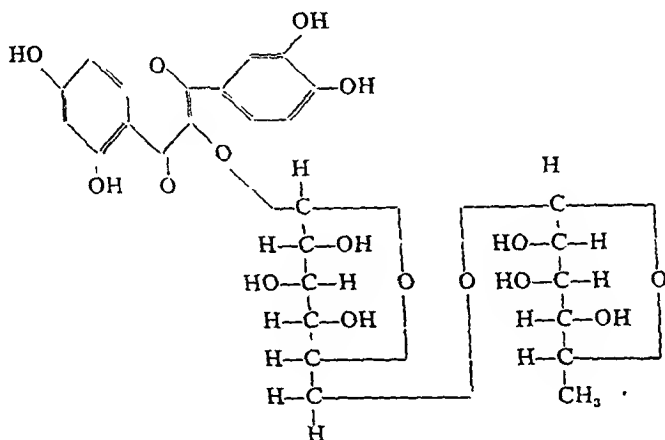
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RUTIN is a flavonol glycoside which is derived from quercetin by condensation of the sugar portion of the molecule with the phenolic hydroxyl group at position 3 of quercetin. On hydrolysis with dilute acid, rutin yields quercetin, rhamnose and glucose in equimolecular proportions.

Rutin has the following formula:



Tentative methods for the determination of rutin and quercetin have been suggested by Porter, Brice, Couch and Copley¹, and their methods have been modified by the Research Division of Penick and Co., New York.² The methods of Porter *et al.* are based on the determination of $E_{362.5}^*$ and E_{375} for rutin and quercetin.

Penick and Co. state that if the ratio $\frac{E_{375}}{E_{362.5}} = 0.875 \pm 0.004$ the quercetin content is reported as being less than 1 per cent., and the percentage of rutin is given by the relation $\frac{E_{362.5} \times 100}{325.5}$ where $325.5 = E_{362.5}$ for pure anhydrous rutin. It will later be shown that $\frac{E_{375}}{E_{362.5}}$ is approximately 0.876 for rutin containing 1 per cent. of quercetin and that the contribution of 1 per cent. of quercetin to the gross absorption is approximately equivalent to that given by 2 per cent. of rutin. Thus in the case of rutin containing 1 per cent. of quercetin the rutin content given by the ratio $\frac{E_{362.5} \times 100}{325.5}$ will be 2 per cent. in excess of the true

*Throughout the paper E_x is used to indicate $E_{\frac{1}{1 \text{ cm.}}}$ at wave length $x \text{ m}\mu$

value. If the ratio $\frac{E_{375}}{E_{362.5}}$ is greater than 0.879, Penick and Co. calculate the rutin content from the formula.

$$\text{Rutin per cent.} = 1.4722 E_{362.5} - 1.3324 E_{375}$$

$$\text{and Quercetin per cent.} = -0.5406 E_{362.5} + 0.6183 E_{375}$$

Further, small differences, within the limits of experimental error in the values of $E_{362.5}$ and E_{375} for rutin and quercetin will alter the formulae with the result that the rutin content as given by the formula is only likely to be correct within ± 0.5 per cent. under optimum conditions.

Both Penick and Co. and Porter *et al.* previously dry the sample at

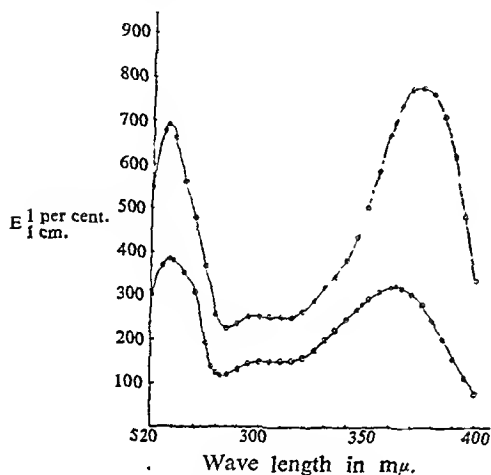


FIG. 1. Absorption spectra

Upper graph—quercetin. Lower graph—rutin

125°C. for 4 and 16 hours respectively in high vacuum before carrying out the assay. If the assay is to be applied to commercial samples such a procedure would necessitate a moisture determination carried out under the same conditions, and the time required to carry out an assay would be considerable. For this reason and the fact that no claims have been made to estimate less than 1 per cent. of quercetin in rutin-quercetin mixtures

it was decided to undertake a systematic study of the problem.

Absorption Spectra of Rutin and Quercetin. When dissolved in alcohol (95 per cent.) containing 1 per cent. of 0.02N acetic acid both rutin and quercetin obey Beer's and Lambert's laws. As shown in Figure 1 rutin exhibits absorption maxima at 259 $m\mu$ and 362.5 $m\mu$ and quercetin exhibits maxima at 257 $m\mu$ and 375 $m\mu$. The maxima at 362.5 $m\mu$ and 375 $m\mu$ are suitable for the determination of rutin and quercetin in mixtures.

Choice of Solvent. It was found that for rutin $E_{362.5}$ increased with increasing alcohol concentration without marked change in the wave-

TABLE I
EFFECT OF INCREASING THE CONCENTRATION OF ALCOHOL ON WAVE LENGTH OF MAXIMUM ABSORPTION

Alcohol Concentration	$E_{362.5}$	Alcohol Concentration	E_{375}
50 per cent.	277	90 per cent.	292
60 "	281	95 per cent.	300
70 "	285	95+1 per cent. of 0.02N	
80 "	288	acetic acid	304

length of maximum absorption. The results in Table I were obtained on a commercial sample.

The use of alcohol (95 per cent.) permits increased accuracy in the determination, since the solubility of rutin in acid alcohol (95 per cent.) is very much greater than in alcohol (50 per cent.). Acetic acid (0.02N) is added to maintain the final dilution on the acid side of neutrality, since mention is made in the literature of the capacity of rutin to form metallic salts.^{3,4}

Hydration of Rutin. If samples of rutin previously dried to constant weight at 110°C. are assayed, the maximum value of $E_{362.5}$ which can be obtained for a sample which is completely soluble in acid alcohol (95 per cent.) is approximately 300. After drying samples at 110°C. in a vacuum below 1 mm. Hg. pressure over phosphorus pentoxide for 2 hours, and assaying the dried sample, it is found that the value of $E_{362.5}$ increases to approximately 325. This corresponds to a loss of 7.7 per cent. of moisture on drying. Rutin is known to exist as the trihydrate and this contains approximately 8.1 per cent. of water of crystallisation. Thus, drying at 110°C. in a high vacuum for 2 hours removes all the water of crystallisation. From the above it seems likely that commercial samples of rutin will consist essentially of rutin trihydrate plus hygroscopic moisture. This has been confirmed for samples so far examined.

Preparation of Pure Rutin. A sample of crude rutin prepared by extraction from buckwheat was purified as follows:

A. The sample was dissolved in alcohol, the least possible quantity of alcohol being used, and the rutin was then reprecipitated by the addition of sufficient water to reduce the alcohol concentration below 10 per cent. After standing in a refrigerator overnight, the rutin was filtered and washed with water.

B. The purified rutin from section A was dissolved in the least possible quantity of alcohol (75 per cent.), warming to effect solution. The solution was cooled, and brown material, which was precipitated on cooling, was filtered off. The alcohol was then distilled off until the point of incipient crystallisation was reached. The solution was allowed to cool, the rutin filtered, washed with small amounts of alcohol (75 per cent.) and dried at 110°C.

C. The filtered rutin was then dissolved in boiling 99 per cent. isopropyl

TABLE II
SPECTROPHOTOMETRIC DATA FOR SAMPLES OF RUTIN AND QUERCETIN
DRIED AT 110°C. IN A HIGH VACUUM

	E_{317}	$E_{362.5}$	E_{375}
<i>Rutin</i>			
Original sample	279.4, 277.8	321.0, 321.8	280.0, 280.7
Sample A	282.5, 282.8	325.2, 325.5	284.9, 283.0
Sample B	281.4, 282.6	324.9, 325.2	282.4, 283.2
Sample C	281.2, 281.0	325.2, 324.5	283.1, 283.0
<i>Quercetin</i>			
Sample D		695, 693	
Sample E	460	705	774, 768
Sample F	458	702	786 780

The values of E_{317} , $E_{362.5}$ and E_{375} for samples C and F did not alter with subsequent treatment.

alcohol, the solution was concentrated, cooled to room temperature, filtered and poured into 10 volumes of hot water. When precipitation was complete the rutin was filtered off.

Preparation of Pure Quercetin. D. Crude quercetin was prepared by refluxing purified rutin with 1 per cent. hydrochloric acid (100 ml./g. of rutin). The quercetin was filtered off and washed with cold water. The crude quercetin was twice crystallised from alcohol (80 per cent.). Samples E and F were taken from the first and second recrystallisations respectively and sample F melted at 313°C. Samples A. B. C. D. E and F were dried at 110°C. in a high vacuum and examined spectrophotometrically with the results given in Table II.

Assay of Rutin and Quercetin in Mixtures. According to Vierordt,⁵ two components of a mixture may be assayed by means of the following formulæ, if values of E for both substances and for the mixture are known for two selected wavelengths.

In the present case

$$\text{Percentage of rutin} = \left(\frac{c_0 b_1 - c_1 b_0}{a_0 b_1 - a_1 b_0} \right) \times 100$$

$$\text{Percentage of quercetin} = \left(\frac{c_0 a_1 - c_1 a_0}{b_0 a_1 - b_1 a_0} \right) \times 100$$

where a_0 = $E_{362.5}$ pure anhydrous rutin.

a_1 = E_{375} pure anhydrous rutin.

b_0 = $E_{362.5}$ pure quercetin.

b_1 = E_{375} pure quercetin.

c_0 = $E_{362.5}$ mixture.

c_1 = E_{375} mixture.

The values of a_0 , a_1 , b_0 and b_1 have been determined experimentally and have been assigned the following values:—

$$a_0 = 325, a_1 = 283, b_0 = 702, b_1 = 780.$$

The equations then become

$$\text{percentage of rutin} = 1.4224 E_{362.5} - 1.2802 E_{375}$$

$$\text{percentage of quercetin} = -0.5161 E_{362.5} + 0.5927 E_{375}$$

The latest formulæ published by Penick and Co. are

$$\text{percentage of rutin} = 1.4722 E_{362.5} - 1.3324 E_{375}$$

$$\text{percentage of quercetin} = -0.5406 E_{362.5} + 0.6183 E_{375}$$

The results in Table III were given on mixtures of known composition, by the Penick formulæ, the formulæ derived above, and by a graphical method which will be described later in the paper.

From Table III it will be seen that in the case of the Penick formula, the percentage of quercetin is correct within the limits of experimental error to be expected in such a determination, whilst the rutin results with one exception are about 1 per cent. high. In the case of the derived formula, the percentage of rutin is approximately correct, whilst the quercetin results are about 0.5 per cent. high.

DETERMINATION OF RUTIN AND QUERCETIN

TABLE III

COMPARISON OF THE RESULTS OBTAINED BY THREE METHODS FOR MIXTURES OF KNOWN COMPOSITION

THEORETICAL		PENICK FORMULA		DERIVED FORMULA		GRAPH	
Rutin per cent.	Quercetin per cent.	Rutin per cent.	Quercetin per cent.	Rutin per cent.	Quercetin per cent.	Rutin per cent.	Quercetin per cent.
99.03	0.97	101.20	0.72	100.00	1.40	99.25	0.75
98.60	1.40	99.67	1.58	98.40	2.20	98.60	1.40
98.08	1.92	98.36	1.61	99.90	1.98	98.05	1.95
97.52	2.48	98.55	2.62	97.08	3.20	97.30	2.70
97.14	2.86	98.37	3.00	97.23	3.60	96.40	3.60
96.59	3.41	97.68	3.47	96.58	4.00	96.30	3.70
96.23	3.77	97.52	3.79	96.43	4.36	96.00	4.00
95.69	4.31	96.54	4.33	95.59	4.88	95.60	4.40
95.53	4.67	96.66	4.54	95.62	5.08	95.30	4.70

The mixtures were made from purified rutin and quercetin previously dried at 110° C. for 2 hours in a high vacuum.

Since the ratio $\frac{E_{347}}{E_{375}}$ is a measure of the quercetin content, it was decided to attempt to use the ratio to measure the quercetin content and hence the rutin content by difference or calculation. Table IV has been constructed from the data for rutin and quercetin.

TABLE IV

MEASUREMENT OF RUTIN CONTENT BY THE RATIO $\frac{E_{347}}{E_{375}}$

Rutin per cent.	Quercetin per cent.	$\frac{E_{347}}{E_{375}}$	$\frac{E_{347}}{E_{362.5}}$
100	0	0.9936	0.871
99	1	0.9828	0.876
98	2	0.9722	0.881
97	3	0.9618	0.886
96	4	0.9520	0.891
95	5	0.9419	0.895
94	6	0.9330	0.900

It will be seen that the ratio $\frac{E_{347}}{E_{375}}$ changes more rapidly with the increased amounts of quercetin than the ratio $\frac{E_{375}}{E_{362.5}}$

The ratio $\frac{E_{347}}{E_{375}}$ has been determined for the mixtures in Table III, and the percentage of quercetin read off from the graph plotted from the data in Table IV. It will be seen that in nearly every case the percentages of rutin and quercetin agree with the theoretical values ± 0.2 per cent. This method is not applicable to the rapid assay of commercial samples, as it would necessitate drying to constant weight in high vacuum at 110°C. However, as the graph will indicate the relative amounts of rutin and quercetin, by means of a formula, samples may be assayed without previous drying.

The following method is proposed for the routine assay of a rutin-quercetin mixture.

Weigh 50 mg. and dissolve in ethyl alcohol (95 per cent.), using 50-ml. graduated flasks. Dilute suitably to give a density reading of 0.4 to 0.6 when the wave length scale of the Beckman spectrophotometer is set at 362.5 m μ . Add 0.5 ml. of 0.02N acetic acid to the last dilution and compare the optical density of this solution with that of a solution of ethyl alcohol (95 per cent.), containing a similar amount of acetic acid, using 1 cm. cells, a tungsten lamp and a Corning No. 9863 Red-Purple Corex A filter.

Calculate E_{347} , $E_{362.5}$, E_{375} and the ratio $\frac{E_{347}}{E_{375}}$

From Table IV or a prepared graph read off the composition of the mixture corresponding to the ratio.

If x = percentage of quercetin read on graph, then rutin: quercetin = $100 - x : x$ or quercetin = $\left(\frac{x}{100 - x}\right)$ rutin.

Let the actual rutin content of sample be y per cent. anhydrous rutin, then quercetin content will be $\left(\frac{x}{100 - x}\right) y$.

For a mixture $E_{362.5} = 3.25 y + 7 y \left(\frac{x}{100 - x}\right)$

Here x and $E_{362.5}$ are known, so y , the anhydrous rutin content of the sample may be calculated.

From this the quercetin is given by $\left(\frac{x}{100 - x}\right) y$ percentage of anhydrous rutin $\times 1.088$ = percentage of rutin trihydrate.

It is important to ascertain that the maximum absorption does not lie on the ultra-violet side of 362.5 m μ . If the maximum is at a shorter

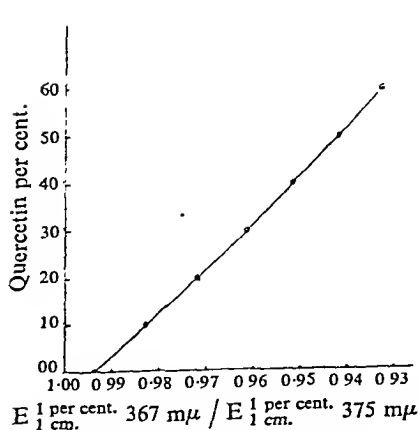


FIG. 2. Percentage of quercetin in mixtures of anhydrous rutin and quercetin.

red pigment have been extracted. Porter *et al*² have given a method

wave length than 362.5 m μ , then the determination will be rendered inaccurate by the presence of other absorbing substances. None of the methods which have been proposed for the assay of rutin is directly applicable if there are considerable amounts of chlorophyll and red pigment present. Chlorophyll and red pigment may be tested for by the extraction of the sample with ether. If chlorophyll and/or red pigment are present, the ether will become coloured. Pure rutin is insoluble in ether. The above method may be applied after the chlorophyll and

DETERMINATION OF RUTIN AND QUERCETIN

for the quantitative determination of chlorophyll and red pigment in rutin.

ASSAY OF RUTIN IN TABLETS

Determine the mean weight of 20 tablets. Powder and mix well. Weigh 50 mg. and dissolve in 80 ml. of ethyl alcohol (95 per cent.), warming to effect solution. Filter and make up to volume with ethyl alcohol (95 per cent.) in a 100-ml. graduated flask. Dilute suitably for the spectrophotometer, adding 1 per cent. v/v of 0.02N acetic acid to the final dilution. Then proceed as instructed for the assay of rutin and quercetin.

$$\text{Mean weight per tablet in mg.} \times \frac{\text{percentage of anhydrous rutin}}{100} \\ = \text{mg. of anhydrous rutin per tablet.}$$

The above assay has been found satisfactory for tablets containing as excipients lactose, starch and gum acacia.

The spectrophotometric measurements in this investigation were carried out on a Beckman Quartz Spectrophotometer calibrated on the mercury lines of wave length 4047Å, 3650Å, 3341Å, and 3132Å.

SUMMARY

1. The ultra-violet absorptions of rutin and quercetin have been investigated.
2. A rapid method has been developed for the accurate determination of the minor constituent of binary mixtures of rutin and quercetin, and a formula derived for the correction of the gross absorption for the absorption due to the minor constituent.
3. A comparison has been made between American methods of assay and the method in use in this laboratory.

The author wishes to express his thanks to the Directors of Allen and Hanburys, Ltd., for permission to publish this paper, to Dr. N. Evers for helpful criticism, and to Mr. W. Smith for providing laboratory facilities.

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THE COLORIMETRIC DETERMINATION OF ANEURINE BY AUERBACH'S METHOD

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AUERBACH'S method¹ for the colorimetric determination of aneurine in pharmaceutical preparations, like that adopted in the United States Pharmacopœia XII, is based on that of Melnick and Field² but is simpler and more rapid than either. Auerbach accelerated the colour development by heating at 60°C. for from 3 to 15 minutes, and for colourless simple solutions of aneurine he diluted with isopropyl alcohol instead of extracting the pigment with xylene, claiming an accuracy of ± 3 per cent. Recently Elvidge³ reported that the method was not reproducible and quoted errors of up to 13 per cent. in determinations without the use of standards; however, even using standards he obtained low and erratic results on tablets, the maximum deviations from the mean being +8 per cent. and -5 per cent. On the other hand Brown *et al.*⁴ reported good agreement between the Melnick and Field colorimetric method and the thiochrome method and pointed out that the former was the more reliable whereas the latter, being more sensitive, was better for samples of low potency. The high sensitivity of the thiochrome method was acknowledged also by Adamson and Handisyde⁵ who reported that in ordinary routine work with this method a greater precision than ± 5 per cent. could not be relied upon. The present paper describes experiments carried out to establish a modified Auerbach's method which was used for control purposes before the thiochrome method became official in the British Pharmacopœia.

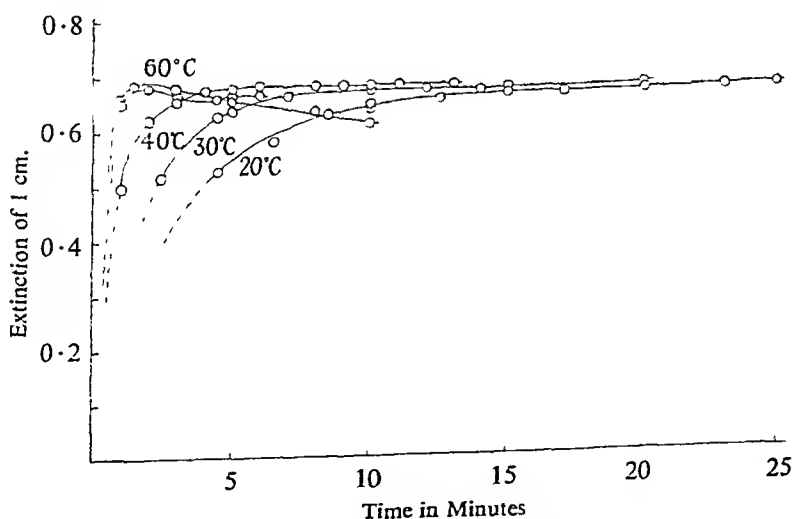


FIG. 1. Rate of Colour Development at Different Temperatures.

COLORIMETRIC DETERMINATION OF ANEURINE

MODIFICATIONS TO THE ORIGINAL METHOD

The original method requires only small volumes of solution and reagents, for example 1 ml. and 2 ml., and an initial modification to increase precision, was to use 5 times the original amounts. However, difficulties in securing uniform heating of the larger volumes were encountered and hence consideration was given to determining conditions under which rapid coupling occurred at room temperature. Figure 1 shows the effect of temperature on the rate of development of colour, 20 to 25 minutes being required for complete coupling at room temperature.

Auerbach specifies the use of 20 to 25 per cent. ethyl alcohol or methyl alcohol as the solvent during coupling, and experiments showed that lower results were obtained with methyl alcohol and isopropyl alcohol but that constant maximum values were obtained with 20 to 25 per cent. ethyl alcohol. For simple solutions Auerbach uses a less alkaline diazotate reagent than that used for an elixir and tablets whereas Allport⁶ recommends the more alkaline reagent for both. Experiments showed that the degree of alkalinity was important, as indeed was expected because of the importance of pH in coupling reactions, and an optimum was chosen. Slightly low results were obtained when the reagent was used immediately after preparation but constant values resulted when the reagent was used between 2 and 3 minutes after adding the alkali. The rate of colour development increased with increase in concentration of the diazotate reagent and, using 0.06 per cent., maximum colour was obtained in 10 minutes. Acid added after coupling slightly decreased the colour intensity, but when added before coupling was complete it arrested the coupling reaction and this effect was used in determining colour development curves.

SOLUTIONS OF ANEURINE

Although according to Auerbach many substances affect the intensity and shade of the colour produced, it was found that traces of inorganic salts and a relatively large proportion of dextrose in the preparations examined made no difference. The reproducibility and precision of the final method were tested by carrying out determinations on different days with freshly prepared reagents (Table I).

TABLE I
REPRODUCIBILITY OF PROPOSED METHOD

Day	Aneurine Solution	Diazotate Reagent	Extinction Value
1	1	A	1.042 1.040 1.044 1.040
2	2	B	1.030 1.033
		C	1.040 1.045
3	2	D	1.041 1.042
		E	1.038 1.041

From these results it was concluded that the modified method, unlike Auerbach's original method, was sufficiently reproducible to permit the use of a calibration curve. The proposed method for simple solutions is described below and calibration curves obtained with International Standard Vitamin B₁ and with a sample of aneurine hydrochloride B.P. are shown in Figure 2.

Reagents. (1) *p*-Aminoacetophenone Solution, 0.06 per cent. in 0.2N hydrochloric acid. (2) Sodium nitrite Solution, 0.2 per cent. (freshly prepared). (3) Diazotate reagent, cool 10 ml. of *p*-aminoacetophenone solution to 5°C., add 3 ml. of sodium nitrite solution and mix. After 3 minutes add 3 ml. of 2N sodium hydroxide and mix by shaking. Use between 2 and 5 minutes after preparation.

Method. Prepare a dilution of the sample to contain about 0.1 mg. of aneurine hydrochloride /ml. and having an acid concentration equivalent to 0.01N. Transfer 5 ml. to a 50 ml. graduated flask, add 10 ml. of ethyl alcohol (50 per cent. v/v), mix, add 5 ml. of diazotate reagent and again mix. Place in a water-bath at 20°C. for 12 minutes and then dilute to 50 ml. with isopropyl alcohol. Determine the extinction in a 1 cm. cell using an Ilford 604 filter, subtract the value of a reagent blank and read off the amount of aneurine from a calibration curve.

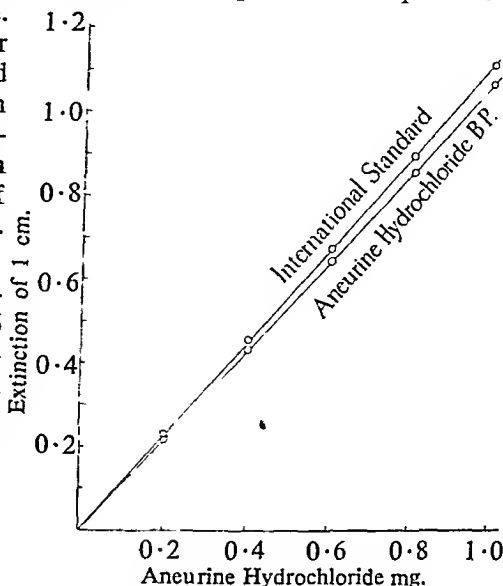


FIG. 2. Calibration curve.

TABLETS OF ANEURINE

Auerbach extracted aneurine from powdered tablets with alcohol (50 per cent.) by heating at 60°C. for 10 minutes. Using this method very low results were obtained (Table II) and this may account for Elvidge's results on tablets which, despite the use of standards, were on an average 20 per cent. low.

TABLE II
EXTRACTION OF ANEURINE FROM TABLETS

Method	Aneurine Hydrochloride found per cent.
Auerbach	1.66, 1.52, 1.54
Alcohol (50 per cent.) at room temperature ...	<div> <div>hours</div> <div> <div>19</div> <div>46</div> <div>146</div> </div> <div> <div>1.88, 1.83</div> <div>2.05, 2.00</div> <div>1.01, 1.76</div> </div> </div>

COLORIMETRIC DETERMINATION OF ANEURINE

Modifications (Table II) gave higher results, but none was considered entirely satisfactory. The United States Pharmacopoeia XII "dissolves" tablets in 0.01N hydrochloric acid whilst Wokes⁷ heats for 10 minutes with a mixture of 15 ml. concentrated hydrochloric acid and 25 ml. of water. For other materials various methods have been used, for example, boiling with 1 per cent. hydrochloric acid⁸ or for 10 minutes with 0.005N hydrochloric acid⁹ and boiling for 1 hour with 0.4N sulphuric acid.¹⁰ Recovery experiments made with the particular tablets under examination showed that the following method was satisfactory.

Method. Finely powder 20 tablets and weigh accurately an amount of powder expected to contain about 10 mg. of aneurine into a 100-ml. conical flask. Add exactly 5 ml. of dilute hydrochloric acid and 10 ml. of water, heat to boiling and boil gently for 4 minutes. Cool, add $(x - 1)$ ml. of N sodium hydroxide, transfer to a 100 ml. graduated flask and dilute to a 100 ml. with water. (The value of x is the number of ml. of N sodium hydroxide required to neutralise the hydrochloric acid in a control determination, after cooling). Use 5 ml. of this solution for a determination as described for simple solutions.

SUMMARY

1. Auerbach's method for the colorimetric determination of aneurine has been modified. The modified method is of greater precision, is reproducible and permits the use of a calibration curve.
2. The inapplicability of Auerbach's method to certain tablets has been shown and a more satisfactory method is described.

Thanks are due to Dr. E. F. Hersant for helpful comments, to Mr. H.W. Johnson for preliminary experiments on tablets, and to the Directors of May and Baker, Ltd., for permission to publish this paper.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Colchicine, Polarographic Determination of. F. Santavy. (*Pharm. Acta Helvet.*, 1948, 23, 380.) The method, applied to the seeds, is as follows. 5 g. of the powdered seeds is macerated for 3 hours at 75°C. with 96.5 g. of water. Water is added to make the weight up to its original value, followed by 3.5 ml. of a saturated solution of lead acetate. After filtration, 60 ml. of the filtrate is treated with 0.25 g. of trisodium phosphate, and again filtered. To 2 ml. of this solution is added 2 ml. of phosphate buffer solution (pH 7 to 8), and, after the removal of oxygen, the polarographic curve is determined. The quantity of colchicine is obtained from a standardisation curve with pure colchicine. On account of the presence of other reducible substances, the values are about 6 per cent. high. For the same reason the method is not suitable for other parts of the plant. For tincture of colchicum, 20 g. of the tincture is evaporated on the water bath to one fourth of its volume and, after cooling, diluted with water to 17 g. The determination is then continued as before with the addition of 1 g. of lead acetate solution and 2 ml. of a saturated solution of sodium phosphate. G. M.

Digitoxin, Effect of Various Alkalis on the Sensitivity of the Baljet Reaction. F. K. Bell and J. C. Krantz, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 297.) The Baljet test, used by the U.S.P. XIII as a colorimetric control for digitoxin, depends upon the red colour produced when a methyl alcoholic solution of trinitrophenol containing sodium hydroxide is added to a methyl alcoholic solution of digitoxin. The effect of replacing the alkali with ammonium hydroxide, lithium hydroxide, tetramethylammonium hydroxide and tetraethylammonium hydroxide was investigated. With ammonium hydroxide the colour was not produced, and lithium hydroxide made no significant difference to the test. The quaternary ammonium bases, on the other hand, gave a deeper, more intense and more stable colour, which also reached its maximum intensity more rapidly. Using these results, the following procedure was developed for preparing the reaction mixture. To 2 ml. of a 0.02 per cent. solution of digitoxin in methyl alcohol, add 0.1 ml. of a 5 per cent. solution of trinitrophenol in methyl alcohol, mix, and add 2 ml. of a 10 per cent. aqueous solution of tetraethylammonium hydroxide. The maximum colour develops in 10 minutes and remains constant, within the limits of experimental error, for about 30 minutes. The modified test is twice as sensitive as that of the U.S.P., and appreciably more sensitive than the alternative Keller-Kiliani test of the U.S.P. G. R. K.

Linoleic Acid in Edible Fats, Determination of. W. J. Stainsby. (*Analyst*, 1948, 73, 429.) The calculation of the composition of a fat containing saturated acids, oleic, and linoleic acids involves the use of 3 simultaneous equations; the total acid equation, another involving the use of iodine values of oleic and linoleic acids, and a third involving the quantitative titration of the acidic glycerides produced by oxidation of the fat. In the third determination the fat is oxidised in anhydrous acetone with potassium permanganate followed by titration of the acidic glycerides

after the removal of the steam-volatile acid products. Results obtained with several hydrogenated cottonseed oils and with 3 samples of sesame, sunflower-seed and palm oils compared very favourably with those obtained by the thiocyanogen method, and by the spectrophotometric method involving alkali isomerisation to a conjugated acid which is subsequently estimated from its ultra-violet absorption spectrum. The method can be extended with little loss of accuracy to determine the total unsaturated acids of oils containing more than 2 unsaturated acids. In the case of fats generally with a higher acid value than that allowed by the British Pharmacopœia, errors arise and such fats should be neutralised before the determination is carried out.

R. E. S.

Methyl Alcohol, Quantitative Colorimetric Microdetermination of, with Chromotropic Acid Reagent. R. N. B o o s. (*Anal. chem.*, 1948, 20, 964.) The reaction of formaldehyde when heated with chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid) in the presence of sulphuric acid to give an intense violet-red colour is used as the basis for the determination of methyl alcohol. A known weight of organic material under test is mixed with water (4 ml.), distilled, and 3 ml. of the distillate collected. One ml. of this solution (diluted to contain 20 to 100 μ g. of methyl alcohol per ml.) is oxidised for 10 minutes with 3 drops of dilute phosphoric acid solution (10 ml. of 50 per cent. acid diluted to 100 ml. with water) and 5 drops of potassium permanganate solution (5 per cent.); decolorisation of excess of potassium permanganate is effected by the addition drop by drop of saturated sodium bisulphite solution. Four drops of a 2 per cent. aqueous solution of chromotropic acid is added, the mixture heated at 60°C. for 15 minutes, cooled in an ice-bath, allowed to reach room temperature and then diluted to 10 ml. The intensity of colour of the solution is measured in a suitable colorimeter, the peak light absorption occurring at 5800Å. A blank determination is necessary each day as the chromotropic acid solution darkens with time. The reaction is specific and the following do not interfere: acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde, isovaleraldehyde, crotonaldehyde, chloral hydrate, glyoxal, benzaldehyde and phthalaldehyde. Glyceraldehyde gives a yellow colour. Good agreement was shown between the method proposed and the Zeisel method, and the method can also be used for the determination of methoxyl groups in methyl esters with a relative error of less than 2 per cent.

R. E. S.

FIXED OILS, FATS AND WAXES

New Zealand Fish Oils. A. P. O l i v e r and F. B. S h o r l a n d. (*Biochem. J.*, 1948, 43, 18.) The fats from 8 specimens of school or snapper shark (*Galeorhinus Australis*) selected at random were studied separately. The livers varied in oil content from 23.1 to 60.7 per cent. and contained from 66.8 to 93.0 per cent. of the total oil reserves of the fish. Tables are given which contain weights of organs and size of each specimen; proportions of tissues and distribution of fats; analytical determinations of vitamin A, unsaponifiable matter, saponification equivalent, and iodine value of liver, body, and head fats. Ester fractionation analyses of 4 of the liver oils, and of the phosphatide and glyceride fractions of the combined head and body lipids showed that the liver fatty acids contained more palmitic acid and C_{18} unsaturated acids, but less stearic acid than the head and body lipids. The wider differences in content of C_{15} , C_{20} and C_{22}

unsaturated acids of the liver oils amounting to as much as 6.3 units per cent. were thought to be outside experimental error, although this was not conclusive in view of previous variations in accuracy. The composition of the liver fats did not appear to be influenced by the extent to which the liver was used for fat storage, in contrast to the results of Rapson obtained from a study of teleostean species.

R. E. S.

Oils and Fats, Stability of. E. Sandell. (*Farm Revy*, 1948, 47, 699, 715.) A low peroxide content of oils and fats is not a guarantee of stability, as in the preparation of the material it is possible that natural antioxidants may have been removed without an appreciable amount of oxidation occurring. Further, traces of metals may have a great influence. Lard has a much lower stability if fish oils have been used in the animal feeding-stuffs. Finally, a strongly oxidised fat may be refined to a low peroxide content, but its stability remains poor. In order to decide on the keeping properties of a sample of oil or fat it is thus necessary to apply special stability tests. In these tests the oxidation is accelerated by raising the temperature, increasing the surface exposed to air, by light, and by traces of metals, of which copper is the most active, while manganese, iron, and chromium also have a marked action. The latter method is of little practical value, since natural antioxidants (synergists) in the oils are probably effective by reason of forming complexes with traces of metals. Accelerated stability tests do not always predict accurately the behaviour of oils and fats on storage, since the reactions may take a different course under different conditions, but they form a useful guide. When testing fatty pharmaceutical preparations, with or without antioxidants, the stability tests should be carried out under conditions approximating as closely as possible to those encountered in actual use. Results obtained by the addition of an antioxidant to a pure fat cannot be extended to a galenical preparation made with that fat.

G. M.

Rape Seed Oil, Component Acids of. M. N. Baliga and T. P. Hilditch. (*J. Soc. chem. Ind., Lond.*, 1948, 67, 258.) The component acids of four rape seed oils—Indian (Toria, Guzerat), Polish (Danzig), and Argentine (Plate)—and of ravison and Jamba rape seed oil have been examined by crystallisation from ether at -40° C. under suitable conditions, previous to ester-fractionation. In this way it was possible to determine approximately the 3 unsaturated and 5 saturated minor component acids as well as the 4 major ones, viz., erucic, oleic, linoleic and linolenic acids. The average fatty acid composition of the 4 rape oils is: palmitic 2.5, saturated C_{18} , C_{20} , C_{22} , C_{24} (together) 5, hexadecenoic 2, oleic 15, linoleic 13.5, linolenic 8, eicosenoic 5, erucic 48, docosadienoic 1 per cent. (wt.). Ravison oil fatty acids contain less erucic (39 per cent.) and more linoleic (21 per cent.). Jamba rape oil fatty acids contain less erucic (37.5 per cent.) and apparently larger proportions of oleic (c. 20 per cent.) and eicosenoic (c. 11 per cent.) acids. The procedure for the examination of the component acids of these cruciferous seed oils, admittedly difficult to resolve, is given in detail.

H. F.

PLANT ANALYSIS

Pyrethrum Flowers, Analysis of. W. Mitchell, F. H. Tresadern and S. A. Wood. (*Analyst*, 1948, 73, 484.) A systematic study has been made of the Seil method, depending on the fact that chrysanthemum

dicarboxylic acid is not volatile in steam in contrast to the monocarboxylic acid, and of the Wilcoxon-Holaday method, depending on the fact that only the monocarboxylic acid is readily soluble in light petroleum. Pure chrysanthemum acids were used in the study and the behaviour, recovery and stability of the acids under varying conditions are reported. The Seil method was found to give low results for pyrethrin I and slightly high results for pyrethrin II, the inaccuracies being due to a temperature effect and not to mineral acid. It is suggested that the apparent loss of chrysanthemum monocarboxylic acid is due to hydration and that the resultant hydroxy-acid is partly responsible for the slightly high figures for pyrethrin II. A modified Seil method gave accurate total pyrethrum figures when compared with the Wilcoxon-Holaday method. The latter method could give accurate results for pyrethrin I and for pyrethrin II if a small modification was used. The methods were applied to pyrethrum extracts confirming the results. The presence of extraneous volatile acids was confirmed but found not to interfere with the accuracy of the results by either method.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Vitamin B₁₂, A Cobalt Complex. E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood and K. Folkers. (*Science*, 1948, 108, 134.) Vitamin B₁₂ appears to be a cobalt co-ordination complex which, having six groups about the cobalt atom, could involve one or more organic moieties. The presence of cobalt is significant in view of the many biological studies which have shown it to be an essential trace element in nutrition. The cobaltous ion (1 µg./ml.) was without activity for *L. lactis* as contrasted with the high potency of B₁₂ (0.000013 µg./ml., half maximum growth). Spectrographic examination of B₁₂ showed the presence of phosphorus; nitrogen was present but tests for sulphur were negative. Microbiological assay of an aqueous solution at B₁₂ (74 µg./0.5 ml.) showed that autoclaving for 15 minutes at 121°C., did not change the activity within the experimental error of $11.4 \times 10^6 \pm 0.6 \times 10^6$ µ/mg. Vitamin B₁₂ in 0.015N sodium hydroxide solution (0.2 µg./ml.) was inactivated (microbiological assay) at room temperature as follows: 20 per cent. (0.67 hr.), 45 per cent. (6 hr.), 90 per cent. (23 hr.), 95 per cent. (95 hr.); it was inactivated in 0.01N hydrochloric acid solution (10 µg./ml.) as follows: 18 per cent. (3 hr.), 75 per cent. (23 hr.), 89 per cent. (95 hr.).

R. E. S.

BIOCHEMICAL ANALYSIS

Myanesin in Body Fluids and Tissues, Determination of. E. Titus, S. Ulick and A. P. Richardson. (*J. Pharmacol.*, 1948, 93, 129.) Two procedures are described. The more useful of these, which is generally applicable to plasma and urine, depends on the fact that under proper conditions phenolic ethers can be made to couple with the more reactive diazonium compounds. A less sensitive method for plasma determinations, involves periodate oxidation of the glycerin side chain to formaldehyde, which may then be determined colorimetrically with chromotropic acid; it gives results in agreement with the coupling procedure. Determination of

plasma levels after intravenous injections into normal dogs of doses of 50 and 100 mg./kg. shows that the compound rapidly disappears from the blood stream. The total amount of myanesin excreted in the urine was in no case more than 2 per cent. of the original dose; it would appear that the drug is distributed throughout the body water.

S. L. W.

Stilbæstrol, Hexæstrol and their Glucuronides in Urine, Colorimetric Estimation of. F. H. Malpress. (*Biochem. J.*, 1948, 43, 132.) Methods of preliminary extraction are described which enable estimations of these æstrogens and their glucuronides in urine to be made, using the nitration method of Malpress. Detailed methods of extraction are given for free stilbæstrol and hexæstrol and for their monoglucuronides in cow's urine; modifications necessary for similar extractions from human urine are also described. It was found that hydrolysis of the urine resulted in destruction of added glucuronide and it was therefore essential to prepare extracts of urines before hydrolysing; traces of peroxide if present in ether used for extraction also reduced the recoveries of the æstrogens. Using the processes described, recoveries ranging from 66 to 79 (mean 70) per cent. were obtained for known amounts of stilbæstrol added to cow's urine, and from 52 to 64 (mean 60) per cent. for known amounts of stilbæstrol glucuronide; recoveries from human urine gave mean values of 80 and 63 per cent. for the free and conjugated forms respectively. The comparatively low recoveries of the glucuronide were due to the decomposition during hydrolysis. With hexæstrol recoveries of 70 per cent. of free æstrogen and 74 per cent. of hexæstrol glucuronide were obtained from cow's urine; for human urine the recovery was 85 per cent. for both free and combined forms. Volumes of urine used for estimation should contain from 0.5 to 2.0 mg. of æstrogen. Blank values for the free æstrogen process using cow's urine fell normally within the range 0.05 to 0.2 mg. of æstrogen/100 ml. of urine, although occasionally these values were greatly exceeded, blanks of 2 mg. being obtained. Blank measurements for the corresponding conjugated-æstrogen method were invariably low and of the order of 0.05 to 0.2 mg. of æstrogen/100 ml. of urine. The values given by the simplified modification applicable to human urine were less than 0.05 mg. of æstrogen for the free process, and less than 0.15 mg. of æstrogen for the conjugated form, from 100 ml. of urine.

R. E. S.

Streptomycin in Tissues and Urine, Chemical Determination of. V. C. Jelinek and G. E. Boxer. (*J. biol. Chem.*, 1948, 175, 367.) The previously reported method of estimation of streptomycin by determining the fluorescence of its acridyl hydrazone has been extended to permit the estimation of streptomycin in body tissues and in urine. The various methods of determining streptomycin are compared and analytical details of the isolation and estimation of streptomycin in lung, brain, heart, liver and spleen tissue and in urine are given. The recoveries of known amounts of streptomycin added to urine and tissues are recorded; they varied considerably. Human urine containing from 2 to 50 µg./ml. gave results of 95 ± 6 per cent.; dog liver gave recoveries of 103 ± 14 per cent.; rabbit brain gave recoveries of 99 ± 6 per cent. The lower limit of sensitivity was 2 µg./ml. of urine and 2 µg./ml. of tissue. The method was found to be of value in the determination of streptomycin in urine and tissues following parenteral administration.

R. E. S.

Suramin in Plasma, Estimation of. J. C. Gage, F. L. Rose and M. Scott. (*Biochem. J.*, 1948, 42, 574.) A method for estimating suramin

in aqueous solution or in serum and plasma is described. The procedure depends on the colour change (deep red to pale yellow) observed when suramin is added to 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride. The corresponding change in absorption is from λ_{\max} 495 $m\mu$ to λ_{\max} 450 $m\mu$ for the two colour bands, with the greatest difference between the two curves at 505 $m\mu$. Plasma proteins do not interfere with the suramin reaction and the concentration of the drug in plasma may be determined by comparing the optical density of the dye solution (at 505 $m\mu$), to which has been added diluted plasma (in sufficient 0.9 per cent. sodium chloride solution to prevent globulin precipitation), with that of the dye solution without suramin. Satisfactory results were also obtained using an absorptiometer with an Ilford 603 blue-green filter. The procedure yields similar results to those obtained using the hydrolysis method followed by diazotisation and coupling with methyl- α -naphthylamine. The hydrolysis products of suramin have been investigated and the specificity of the method is discussed.

R. E. S.

CHEMOTHERAPY

Dienœstrol and Hexœstrol, Tetra-alkyl Substituted Analogues of. J. B. Niederl and P. Weiss. (*J. Amer. chem. Soc.*, 1948, 70, 2894.) By conversion of the phenols, *p*-xylenol, thymol and carvacrol into the corresponding 2:5-dialkyl-4-hydroxypropiophenone, tetra-alkyl analogues of dienœstrol and hexœstrol were prepared. The tetra-alkylated hexœstrols derived from thymol and carvacrol showed only feeble œstrogenic activity when injected subcutaneously in oily solution into ovariectomized rats. The compound derived from *p*-xylenol, 3:4-bis(2':5'-dimethyl-4'-hydroxyphenyl)-hexane, in contrast, gave positive œstrus response in all the rats at 50 and at 5 μg . dose levels and in most of the rats at 2 and 1 μg . dose levels thus comparing favourably with its dimethyl analogue, 3:4-bis-(5'-methyl-4'-hydroxy-phenyl)-hexane prepared from *o*-cresol.

F. H.

Sulphones: Studies in the Chemotherapy of Tuberculosis. E. Hoggarth and A. Martin. (*Brit. J. Pharmacol.*, 1948, 3, 146.) The testing of a large number of sulphones and related sulphonates and sulphonamides against *M. tuberculosis in vitro* is recorded. On the basis of high *in vitro* activity ten new compounds were selected for therapeutic tests on mice. Therapeutic activity was found with 4:4 diaminodiphenylsulphone and with 2:4' diamino-5-thiazylphenylsulphone, but no activity was observed with any of the others. Therapeutic tests in mice show that high *in vitro* activity does not necessarily lead to activity *in vivo*.

S. L. W.

Thiohydantoins and Thioimidazoles. M. Jackman, M. Klenk, B. Fishburn, B. F. Tullar and S. Archer. (*J. Amer. chem. Soc.*, 1948, 70, 2884.) As it had previously been shown that 2-thiohydantoin possessed half and 2-thioimidazole one and a half times the anti-thyroid activity of 2-thiouracil, and that enhancement of activity occurs on substitution of 2-thiouracil, a series of 5-alkyl-2-thiohydantoins and 4-alkyl-2-thioimidazoles has been prepared and examined. It was found that substitution in the 5-position did not result in any significant increase in the anti-thyroid activity of 2-thiohydantoin. In the 2-thioimidazole series, substitution in the 4-position increased the activity, 4-*n*-propyl-2-thioimidazole, the most active compound prepared, being about three times as active as 2-thioimidazole and about five times as active as 2-thiouracil.

F. H.

PHARMACY

DISPENSING

Oils and Fats, Sterilisation of. J. Kessler. (*Pharm. Acta Helvet.*, 1948, 23, 387.) Oils and fats cannot be sterilised either in free steam or in the autoclave. Dry heating (90 minutes at 160°C.) is satisfactory.

G. M.

Sterilisation Technique, Efficiency of. O. Bang and A. T. Dalsgaard. (*Arch. Pharm. Chemi.*, 1948, 55, 699.) For testing the efficacy of various methods of sterilisation, the authors used garden soil. This material required at least 20 minutes at 120°C. to produce complete sterility. The results of the tests showed that in 61 per cent. w/w alcohol, heating at 100°C. for at least 1 hour in a sealed container was necessary for complete sterilisation. When suspended in oil, the dry heating required was at least 10 hours at 140°C., 3 hours at 160°C., or 1 hour at 180°C. The alcohol method may be applied to the sterilisation of procaine hydrochloride and of boric acid in powder, also to laminaria. In the latter case the material is kept under 61 per cent. alcohol for 24 hours to extract soluble salts, then transferred to tubes, covered with the diluted alcohol, and closed with cotton wool and a loosely screwed-on lid. After 1 hour in flowing steam, the alcohol is removed and the tubes are dried at 105°C.

G. M.

PHARMACOLOGY AND THERAPEUTICS

Conessine, isoConessine and neoConessine, Pharmacological Properties of. R. P. Stephenson. (*Brit. J. Pharmacol.*, 1948, 3, 237.) Conessine, an alkaloid obtained from the bark and seeds of *Holarrhena antidysenterica*, and its isomers, *isoconessine* and *neoconessine* (prepared by treating conessine with sulphuric acid) possess properties very similar to those of quinine and quinidine; in doses, however, in which quinine was active as an antimalarial, conessine and its isomers showed no similar activity. When tested by intracutaneous injection into guinea-pigs conessine and its isomers were shown to possess marked local anæsthetic potency, conessine being about twice as active as cocaine, *isoconessine* about 50 per cent. stronger than cocaine, and *neoconessine* being about equal to cocaine. The relative local anæsthetic potencies of conessine and its isomers and of cocaine, quinidine and procaine are very similar to their related activities in depressing the action of acetylcholine on the frog rectus muscle. This is a further addition to the evidence that the action of acetylcholine is concerned with the sensation of pain. Conessine also resembles quinidine in diminishing the action of acetylcholine on the isolated intestine, the rabbit auricle, the frog rectus and on denervated mammalian muscle, and in lengthening the refractory period of cardiac tissue, and the effect of vagal stimulation on the heart in the anæsthetised rabbit is temporarily abolished by conessine as by quinidine.

S. L. W.

Dimercaprol (B.A.L.), Effect of Environmental Temperature on. F. F. McDonald. (*Brit. J. Pharmacol.*, 1948, 3, 116.) Variations of 15 to 85 per cent. in mortality occurred in groups of rats used as standard controls in assaying samples of dimercaprol. So wide a difference in response was greater than would be expected by chance and some other external factor was suspected of contributing to the toxic effects of dimer-

caprol. Temperature being the greatest variant to which the rats were subjected, experiments were conducted to see if this affected the mortality of rats caused by a standard dose of dimercaprol. Rats injected intramuscularly with 140 mg./kg. of Oxford Standard B.A.L. were kept in thermostatically controlled chambers at temperatures varying from 39°F. to 84°F. for 72 hours after injection. The results showed a quite remarkable effect of temperature on toxicity, the mortality rate varying from 20 to 100 per cent., with a minimum mortality at 63°F. Rats used for assays should therefore be kept at an even temperature or in a thermostatically controlled room if possible, and no estimation of the relative toxicity of samples should be made without reference to the results obtained from a dose of a standard preparation given at the same time. If propylene glycol is used as a vehicle for injection, the required amount should be distilled off on the same day.

S. L. W.

Dimercaprol. (B.A.L.) and its Glucoside, Effects of, in Acute Lead Poisoning. M. Weatherall. (*Brit. J. Pharmacol.*, 1948, 3, 137.) In mice poisoned by repeated intraperitoneal injections of lead acetate the mortality was reduced slightly by dimercaprol and significantly by the glucoside, but it was difficult to produce lead poisoning suitable for experimental study in these animals. In suitable concentrations dimercaprol prevented the action of lead acetate on rabbit red blood cells *in vitro*. If it was added half or one hour after the lead acetate the effect was small and consisted chiefly in preventing the full effect of the lead, not of significantly reversing the established change in fragility. Mixtures of dimercaprol and plasma in certain proportions, and plasma from rabbits injected with dimercaprol, protected washed erythrocytes from the effect of lead acetate less than did equal amounts of dimercaprol or plasma alone. In rabbits poisoned by a single dose of lead acetate given by stomach tube dimercaprol and the glucoside each significantly decreased the subsequent anæmia and increased the coproporphyrinuria. The mortality was apparently unaffected by dimercaprol but was reduced by the glucoside, though the number of rabbits was too small for the difference in mortality to be significant. The action in lead poisoning appears to be one of inactivating lead ions not yet taken up by cells, rather than of actually de-leading cells or altering the cell lead so as to prevent its fragility effect. There is certainly no reversal of poisoning comparable to that seen with arsenicals. Clearly the drug prevents the acute hæmolytic anæmia as long as it is available in the circulation, but on the other hand certain features of lead poisoning are enhanced, notably the coproporphyrin excretion and possibly the speed of the reticulocyte response. The available evidence does not warrant its use in clinical plumbism, but it would perhaps be premature to reject all dithiols as useless or dangerous.

S. L. W.

Iron, Intravenous, in the treatment of Anæmia. H. G. B. Slack and J. F. Wilkinson. (*Lancet*, 1949, 256, 11.) A stable iron-sucrose preparation suitable for intravenous administration was prepared as follows. Dissolve anhydrous ferric chloride 5.8 g. in distilled water 50 ml. on a water-bath at 95°C, add sucrose 28 g. and heat until dissolved; dissolve anhydrous sodium carbonate 1.8 g. and sodium hydroxide 5 g. in 25 ml. of water each; add the carbonate solution to the ferric chloride solution with stirring; add the sodium hydroxide solution, stir for 15 minutes, filter into rubber-capped vials or into ampoules and autoclave for 20 minutes. After autoclaving, a clear, dark brown solution is obtained, with a pH of about 10.5, which remains stable at room temperature for at least 12 months. The solution contains

2 per cent. of iron and 200 mg. is therefore contained in 10 ml. The scheme of dosage adopted in the treatment of 60 cases of iron-deficiency anaemia was 25 mg. on the first day, 50 mg. on the second day, 100 mg. on the third, and 200 mg. on the fourth and subsequent days, the injections being given into the antecubital veins at the rate of about 2 ml. per minute; the treatment was usually complete in 10 out-patient visits. Reactions, if any, were mild. In almost all cases the hæmatological and clinical responses were often as dramatic as those obtained when patients with pernicious anaemia in relapse receive adequate doses of a potent intramuscular liver extract, a reticulocyte peak of 10 to 18 per cent. developing within 7 to 10 days from the beginning of treatment. The calculated iron deficit given in this manner is utilised almost quantitatively and does not appear to require the addition of trace elements, ascorbic acid or folic acid. With a further 60 patients treated with a commercial iron-sucrose preparation there was no detectable difference in tolerance or response.

S. L. W.

Paludrine, Activation of. F. HAWKING and W. L. M. PERRY. (*Brit. J. Pharmacol.*, 1948, 3, 320.) Experiments with exo-erythrocytic forms of *Plasmodium gallinaceum* grown on tissue culture showed that paludrine in concentrations of 2 mg./l. exerts no apparent antimalarial action on the parasites *in vitro*; similarly, a concentration of paludrine of 20 mg./l. has no action *in vitro* on the endo-erythrocytic form of *P. cynomolgi*; these concentrations are higher than those commonly reached in the blood during human therapy. If, however, paludrine has been previously exposed to the action of body cells, either by injecting it into a monkey or fowl and collecting the serum, or by incubating it with minced rat liver, it exerts marked antimalarial action, preventing the development of the parasites. These results suggest that paludrine itself is not active against plasmodia, but that it undergoes some chemical modification by the body or by liver which converts it into a compound with plasmodicidal activities.

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H. T. B.

Posterior Pituitary Extract, Standardisation. Modification of Dale and Laidlaw Method. P. HOLTON. (*Brit. J. Pharmacol.*, 1948, 3, 328.) The Dale and Laidlaw method suffered from three defects, namely, that suitable guinea-pigs were relatively scarce, that the assay often required many hours, and that the error was about 20 per cent. Schild's null hypothesis (*J. Physiol.*, 1942, 101, 115) was applied to an assay employing a modification of the Dale and Laidlaw method. A rat's uterus was used as the test preparation, since rats are cheaper and more easily obtained. One assay is described, and the

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PHARMACOPŒIAS AND FORMULARIES

THE BRITISH PHARMACOPŒIA 1948 SOME OBSERVATIONS ON THE TESTS FOR PURITY

By H. BAGGESGAARD-RASMUSSEN.

*Professor of Organic Chemistry in the Danish Pharmaceutical College, Copenhagen
Member of the Danish Pharmacopœia Commission*

IN the official tests for degree of purity, and the methods of assay for chemicals, the new British Pharmacopœia shows remarkable progress when compared to the previous one (1932). Throughout the book the formulation is more precise, the tests are more comprehensive, and frequently more rational; and many new methods have been introduced.

Determination of melting-points.—The technique—the capillary method—is that generally described in modern pharmacopœias, and is completely identical with the method used in the Pharmacopœia of 1932. The melting temperatures may be regarded as corrected temperatures consistent with the technique employed. On the other hand the definition, viz.: The temperature at which liquefaction of the substance occurs; this is indicated by the formation of a definite meniscus, may be subject to criticism, because it is expressed by a single temperature and not by a range between two temperatures. In general, commercial products are rarely of such purity that their melting-points are sharply defined; usually the melting-range extends over one or two degrees. The United States Pharmacopœia uses the term melting-range or melting-temperature, which is thus defined "The temperature at which the column of the sample (in the capillary tube) is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of the melting." Many other Pharmacopœias (e.g. Danish, Swiss, Swedish) give a similar definition.

Determination of boiling-points.—Both the apparatus used in the determination of the boiling-point and the procedure are described in detail, as in the Pharmacopœia of 1932. The method is the conventional one, as employed in, e.g. the test for oils, and it is well known that this gives reproducible results, although the use of a cylindrical flask as described respectively in the Swiss and the new Danish Pharmacopœias is more rational.^{1,2} It is a disadvantage that the procedure demands a large quantity of the substance, 100 ml. In most cases the procedure is employed to decide the character of mixtures of compounds as, for instance, creosote and cresol. It would have been an advantage if the technique in the determination of substances with low boiling-point as e.g. cyclopropane (-34.5°C.) and ethyl chloride (about $+12.5^{\circ}\text{C.}$) had been described in detail.

The determination of the boiling-point in the manner described can scarcely be regarded as a practical test for identity and such a test might be useful in many cases, e.g. amphetamine. A number of methods requiring small quantities only and giving reproducible results are available for that purpose; the method described by Siwoloboff,³ and modifications,⁴ may be mentioned.

Limit test for chlorides.—In practically every relevant instance the limit test for chloride is used; although vague expressions such as "no opalescence occurs immediately" have not been completely eliminated. The limit test is carried out in Nessler glasses using 50 ml. of the solution to be tested; to this is added 1 ml. of nitric acid and a solution of silver nitrate (N/10); then the mixture is stirred with a glass rod, and the observation of the reaction

2 per cent. of iron and 200 mg. is therefore contained in 10 ml. The scheme of dosage adopted in the treatment of 60 cases of iron-deficiency anaemia was 25 mg. on the first day, 50 mg. on the second day, 100 mg. on the third, and 200 mg. on the fourth and subsequent days, the injections being given into the antecubital veins at the rate of about 2 ml. per minute; the treatment was usually complete in 10 out-patient visits. Reactions, if any, were mild. In almost all cases the hæmatological and clinical responses were often as dramatic as those obtained when patients with pernicious anaemia in relapse receive adequate doses of a potent intramuscular liver extract, a reticulocyte peak of 10 to 18 per cent. developing within 7 to 10 days from the beginning of treatment. The calculated iron deficit given in this manner is utilised almost quantitatively and does not appear to require the addition of trace elements, ascorbic acid or folic acid. With a further 60 patients treated with a commercial iron-sucrose preparation there was no detectable difference in tolerance or response.

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Determination of melting-points.—The technique—the capillary method—is that generally described in modern pharmacopœias, and is completely identical with the method used in the Pharmacopœia of 1932. The melting temperatures may be regarded as corrected temperatures consistent with the technique employed. On the other hand the definition, viz.: The temperature at which liquefaction of the substance occurs; this is indicated by the formation of a definite meniscus, may be subject to criticism, because it is expressed by a single temperature and not by a range between two temperatures. In general, commercial products are rarely of such purity that their melting-points are sharply defined; usually the melting-range extends over one or two degrees. The United States Pharmacopœia uses the term melting-range or melting-temperature, which is thus defined "The temperature at which the column of the sample (in the capillary tube) is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of the melting." Many other Pharmacopœias (e.g. Danish, Swiss, Swedish) give a similar definition.

Determination of boiling-points.—Both the apparatus used in the determination of the boiling-point and the procedure are described in detail, as in the Pharmacopœia of 1932. The method is the conventional one, as employed in, e.g. the test for oils, and it is well known that this gives reproducible results, although the use of a cylindrical flask as described respectively in the Swiss and the new Danish Pharmacopœias is more rational.^{1,2} It is a disadvantage that the procedure demands a large quantity of the substance, 100 ml. In most cases the procedure is employed to decide the character of mixtures of compounds as, for instance, creosote and cresol. It would have been an advantage if the technique in the determination of substances with low boiling-point as e.g. cyclopropane ($-34.5^{\circ}\text{C}.$) and ethyl chloride (about $+12.5^{\circ}\text{C}.$) had been described in detail.

The determination of the boiling-point in the manner described can scarcely be regarded as a practical test for identity and such a test might be useful in many cases, e.g. amphetamine. A number of methods requiring small quantities only and giving reproducible results are available for that purpose; the method described by Siwoloboff,³ and modifications,⁴ may be mentioned.

Limit test for chlorides.—In practically every relevant instance the limit test for chloride is used; although vague expressions such as "no opalescence occurs immediately" have not been completely eliminated. The limit test is carried out in Nessler glasses using 50 ml. of the solution to be tested; to this is added 1 ml. of nitric acid and a solution of silver nitrate (N/10); then the mixture is stirred with a glass rod, and the observation of the reaction

is made 5 minutes later. The opalescence produced is compared to a standard opalescence, equivalent to 0.355 mg. Cl⁻/50 ml. (0.071 mg. Cl⁻/ml.).

The limit test for chlorides has been carefully investigated by Thörn⁵ and by Reimers and Gottlieb⁶. The results of these investigations may be summarised as follows. The opalescence produced by minute quantities of chloride is in inverse ratio to the speed at which the reagent and the test solution are mixed together. Thus rapid mixing does not lead to the maximum sensibility, but gives the most reproducible results. If the reagent is added without shaking, it will give the maximum of sensibility, but with poor reproducibility. For instance the opalescence produced when the solution of silver nitrate is added to the solution to be tested and the liquid shaken after 1 minute, is about 3 times more intense than that produced after rapid mixing. The procedure as set out in the B.P. gives good reproducible results.

Limit test for sulphates.—The test for sulphates is a simple one. Dissolve the substance in water and transfer to a Nessler glass; add hydrochloric acid; dilute to 50 ml. with water, and add 1 ml. of solution of barium chloride. Stir immediately with a glass rod and set aside for 5 minutes. The turbidity produced is then compared to a standard turbidity equivalent to 2.4 mg. of SO₄/50 ml. (0.048 mg./ml.). The conditions here are not quite so simple as in the limit test for chlorides. Thörn⁷ and Reimers and Gottlieb⁸ have proved that to obtain good reproducibility, it is necessary for the solution to contain a minute quantity of scarcely visible crystals of barium sulphate. Actually the solubility of barium sulphate increases with the decrease in size of the particles owing to the greater surface energy of the small particles. The precipitation of barium sulphate from very dilute sulphate solutions must commence with the formation of microcrystals, the solubility of which may be as much as 1000 times greater than that of large barium sulphate crystals. The precipitation will be markedly inhibited unless the test is carried out with a reagent containing barium sulphate to induce the precipitation in the test sample. The Danish Pharmacopœia describes the use of such a seeding reagent—the same quantities of sulphate in the test and in the standard turbidity—the concentration of which is chosen so that the precipitation is rapidly completed. The application of the seeding reagent results in both an increased sensibility and an increased reproducibility. The simple method of the B.P. cannot be said to lead to exact reproducible results.

Limit test for iron.—The limit test for iron is now carried out with thioglycollic acid in a solution containing citric acid to produce complex compounds with other cations, and an excess of ammonia to make the solution alkaline. In the presence of iron a pinkish-violet colour is produced. This is a notable improvement on the thiocyanate method formerly employed. Woods and Mellon⁹ state that the thiocyanate method in general is inferior to several other methods, especially those using *o*-phenanthroline, and $\alpha\alpha$ -dipyridyl or thioglycollic acid.

Thioglycollic acid as a reagent for iron was first proposed by Andreasch¹⁰ and has subsequently been investigated by others, of whom Swank and Mellon¹¹ state that the thioglycollic acid method is remarkably free from the influence of other common anions, many of which must be entirely absent in other colorimetric methods. The following ions, in concentrations of 500 mg./100 ml. of solution, had no effect on the colour:—fluoride, iodide, nitrate, orthophosphate, sulphate, chlorate, tartrate, oxalate, citrate, acetate, bromide, thiocyanate, sulphite, and chloride: 250 mg. of boron trioxide, present as tetraborate ion, also has no effect. Pyrophosphate ion, when present in an amount equivalent to 500 mg. of phosphorus pentoxide decreases

the colour intensity by about 8 per cent., but 200 to 300 mg. can be present without serious error. Cyanide ion interferes seriously and must be absent. The lack of interference by nearly all anions and the reproducibility and sensitivity of the colour reaction makes the method superior to various other colorimetric determinations of iron. The procedure is just as simple as the thiocyanate method. The standard colour is equivalent to 0.04 mg. Fe/50 ml. (0.0008 mg./ml.)

Reaction.—For the determination of the reaction of a solution various indicators are used. It is not quite clear what intervals in the pH scale are covered by the designations, strongly acid, weakly acid, neutral, weakly alkaline, and strongly alkaline. Many pharmacopœias use similar terms to describe defined intervals of the pH scale. In many cases it would be more decisive to determine the pH value, or the interval between two pH values as a means of characterising the degree of purity. As the B.P. gives a full description of the colorimetric determination of pH values and the standard buffer solutions for preparing solutions with pH 1.2 to 10.0 such an indication might easily have been inserted more generally and more consistently. It must be regarded as a disadvantage that no test for the sensitivity of litmus paper is given, because the commercial grades of litmus paper vary considerably in this respect. The U.S.P. and the new Danish Pharmacopœia have specified tests, both for sensitivity and for the content of buffer substances in the paper.

Limit tests for metals.—The limit tests for lead and arsenic do not differ much from the previous Pharmacopœia. The expression of the limits in parts per million might well be introduced more generally into other Pharmacopœias. For lead the diphenylthiocarbazone method is used in some cases. It must be regarded as an advance that the B.P. specifies the tests for various individual metals (e.g. copper, zinc) and only in a very few cases uses the general term "heavy metals," which is used in some other Pharmacopœias, e.g. in the Danish, Swedish, Swiss, and U.S.P.

The test for arsenic takes the form used in the British Empire and U.S., while the modern continental Pharmacopœias use the hypophosphite test, which is simpler in technique and equally accurate.

Readily carbonisable substances.—The deletion of the test for readily carbonisable substances is comprehensible in a country where supplies are generally of great purity. One thing is certain, that, if the test is to be of any value, it is essential to have a series of matching fluids at one's disposal as given in the U.S.P. and in the new Danish Pharmacopœia. In the latter, the matching fluids are used not only in this test but generally to determine the colour of many faintly tinted solutions, also for some colorimetric determinations, for instance morphine in codeine and papaverine and for the colorimetric determination of the concentration of adrenaline in solutions.

As an exception, the B.P. includes a test for carbonisable substances in liquid paraffin and similar products. In these cases the colour which is produced in sulphuric acid, after shaking with the paraffin, is measured by means of standardised coloured glasses in accordance with the system of colour measurement adopted at the National Physical Laboratory, Teddington. Such standardised glasses might advantageously be used instead of matching fluids to measure the colour in the test for readily carbonisable substances and also for other approximate colorimetric measurements.

The tests for identification and purity must be said to meet all reasonable demands, a comment equally applicable to the assays. The tests for purity are not numerous, but are adequate for practical purposes, to ensure com-

pounds of sufficient purity. As a large number of new substances have been included a few of the tests and assays will be mentioned.

Ultraviolet absorption.—The absorption of ultraviolet light as a test covering both purity and identity has been included for the following substances: ascorbic acid, ethisterone, calciferol, dieneœstrol, œstrone, progesterone, and the following drugs containing vitamin A: halibut-liver oil, concentrated solution of vitamin A, concentrated solution of vitamins A and D, and cod-liver oil. The extinction coefficient is referred to a 1 per cent. w/v solution and indicated for a given wave-length. For the practical purpose to which it is here applied this indication is more suitable than the molecular extinction coefficient. In some cases it might have been of value to give the absorption not only in the maximum but also in the minimum of the extinction curve.

The identification of substances which are so expensive that only small amounts are available are carried out by melting-point determinations on the pure substances or simple derivatives of them, these tests likewise are satisfactory. Among the more modern tests for identification the cyanogen bromide test for nicotinic acid and nicotinamide may be mentioned. For the determination of iodine ion the titration using potassium iodate in presence of potassium cyanide is used; this is an easy and reliable method, which is used also for determination of iodide in iodoxy after hydrogenation with zinc dust and glacial acetic acid and in iodophthaleim after destruction by heating with anhydrous sodium carbonate. For the determination of iodine in thyroid the powder is heated with sodium carbonate and the iodine ion oxidised to iodate and titrated in the usual way.

For the qualitative test for organically bound chlorine reduction by sodium and amyl alcohol and subsequent titration of the chloride ion formed has been substituted; a more rational method than the old one. This test is used for benzoic acid, benzaldehyde, mandelic acid and its calcium salt, and vinyl ether.

For the determination of the content of bismuth in bismuth salts the old method of ignition is used in several cases, but in some the determination of bismuth as bismuth phosphate is used. This latter procedure is an excellent method and might have been adopted more widely, especially for the salicylate and subgallate, in both of which cases the ignition is protracted.

The assay of organic compounds, which cannot be titrated, in many cases proves difficult. In some cases the determination of the content of nitrogen solves the problem. This method might also advantageously have been used for barbitone. For hexabarbitone and the sodium salt the determination of the double bond in the same way as the iodine value would have been a good assay.

The determination of alkaloids in alkaloidal salts has been discussed by van Os¹². Here it should be sufficient to mention that the principle of weighing or titration of the base is always used in the B.P. and this is the rational way, although other pharmacopœias use only the determination of the anion.

The titrations with titanous chloride for some chemicals (menaphthone, methylene blue, crystal violet) are good.

For the determination of theobromine the excellent method of methylating and weighing the caffeine formed is used. For methylthiouracil no really satisfactory test for identification has been given, as the assay mentioned is inadequate for identification.

The Pharmacopœia contains a large number of Appendices which seem to be comprehensive and very satisfactory. Appendix I gives a list of

materials and solutions employed in tests, these describe the usual reagents, with a complete description of tests for identification and purity.

The solutions of reagents are also listed. It is regrettable that the respective concentrations are not given in simple molarity, but always in per cent. This is an unpractical and old-fashioned way. At least the concentrations of commonly used acids, bases, and some salts ought to be given in simple molarity as is the case in e.g. the Danish, Netherlands, Swedish, and Swiss Pharmacopœias, but, strangely enough, not in the U.S.P.

Appendix V, qualitative reactions and tests for substances mentioned in the Pharmacopœia, gives briefly but exhaustively most of the common identity tests.

The form of the individual monographs is clear, practical and well arranged, and all in all, the new Pharmacopœia is a great improvement on the previous one.

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ABSTRACTS (continued from page 342)

results calculated by Schild's method. Eight satisfactory assays were performed and the mean percentage error was 2.16. Fiducial limits for the estimate of potency were calculated and were found to be about half the value for the original Dale and Laidlaw method. The mean experimental time for an assay was 3½ hours.

S. L. W.

Sulphaguanidine, Absorption of. M. R. Fabre, M. T. Régnier and M. E. Grasset. (*Ann. pharm. Franc.*, 1948, 6, 205.) The method of investigation previously applied to the absorption of sulphanilamide has been extended to sulphaguanidine. After a feed rich in fats dogs were anaesthetised with somnifen and a sample of chyle was collected by catheter. A dose of 5 g. of sulphaguanidine was then given into the stomach. The chyle and blood was examined at intervals for the presence of the drug. The results show that sulphaguanidine is absorbed more slowly than sulphanilamide. In the blood, the compound was first detected after 20 minutes, reaching a maximum after 6 hours. The maximum concentration in the blood was 2.2 mg./100 ml., which compares with the figure of 10.5 mg./100 ml. previously obtained for sulphanilamide. In the chyle, the first signs were detected after 70 minutes, reaching a maximum of 1.9 mg./100 ml. at 5 hours. On post mortem examination, no sulphaguanidine was recovered from the organs, the main quantity being in the urine (13 mg./100 ml.) and faeces (1.20 g./100 g.)

G. M.

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NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Alepsal* is a combination of phenobarbitone, caffeine and belladonna for use in the treatment of epilepsy. It is claimed that the drugs exert a synergistic effect and that this prolongs the action, enables the use of a smaller dosage of barbiturate, and permits of continuous treatment without risk of toxic reactions or depression of the mental and physical condition of the patient. Alepsal tablets are issued in three strengths: Normal, containing phenobarbitone 1.54 g., belladonna 0.31 g., and caffeine 0.38 g.; Medium, half these dosages; Weak, phenobarbitone 0.23 g., belladonna 0.046 g., and caffeine 0.057 g. The dosage for adults is 2 tablets of Normal strength twice daily, or 1 or 2 tablets of Medium strength for mild cases, with smaller doses of Weak strength for children. The tablets are issued in tubes of 20 (Normal), 30 (Medium) and 80 (Weak).

S. L. W.

Avloprocil* is a proprietary brand of procaine-penicillin oily injection, containing 300,000 units of penicillin and 120 mg. of procaine base in each ml. It is claimed that following a single intramuscular injection of 1 ml. effective blood levels can be demonstrated for 18 to 24 hours. The indications are the same as for penicillin oil-and-beeswax preparations, and it is thus of value in all infective conditions where maintained levels of penicillin in the blood are required, and where frequent injections are undesirable or inconvenient. It is of particular value in the out-patient treatment of gonorrhoea by single injection. A single intramuscular injection of 1 ml. (300,000 units) is given every 24 hours, but for severe infections 2 ml. may be necessary. In the treatment of syphilis a course of 1 or 2 ml. daily for 7 or 8 days is given either alone or in conjunction with arsenic or bismuth or both. Under normal storage conditions it retains its potency for at least 12 months. It is issued in single vials containing 10 ml., or in boxes of 5 vials containing 10 ml.

S. L. W.

Cresatin* is the acetic ester of metacresol, and has antiseptic, fungicidal and analgesic properties. Its low volatility and oily character cause it to adhere to tissue surfaces, thus providing prolonged action. It is used in infections of the nose, throat and ear, and has been found of value in the treatment of ringworm of the feet; it may also be used in dental practice in the treatment of infected root canals and sockets. In most conditions it may be used undiluted, except when applied in the form of a saturated absorbent cotton packing to the auditory canal when it should be diluted with 3 or 4 parts of warm olive oil. It is supplied in 1-oz. bottles.

S. L. W.

Crystodigin* is a brand of crystalline digitoxin, 1 mg. producing the same effect by mouth as 1 g. of digitalis. The indications are the same as those for digitalis, its greatest value being exhibited in congestive heart failure, auricular fibrillation, and auricular flutter. Rapid digitalisation may be produced by a single dose of 1.2 mg., the effect being established within 6 hours or less. The maintenance dose is 0.2 mg. daily, though individual adjustment of this dose may be necessary, and may vary from 0.1 to 0.3 mg. daily. For slower digitalisation, daily doses of 0.2 to 0.6 mg. for several days are administered. For patients who have recently received digitalis

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BOOK REVIEWS

A MANUAL OF PHARMACOLOGY by Torald Sollman. 7th Edition, Pp. 1132 and Index. W. B. Saunders Company, London and Philadelphia, 1948, 57s. 6d.

In pharmacology the present decade is one of "anti-drugs." During recent years, pharmacologists and chemists have achieved astonishing results by the development of poisons which act selectively upon one species of living organism or upon one particular type of cell, enzyme or end-organ. The application of the principle of selective poisoning of species has given us new rodenticides, insecticides and weed-killers, the antibiotics, the anti-malarials, antrycide and other antimicrobials. In functional pharmacology, selective poisoning of certain types of cells, enzymes or end organs has given us antihistamine, antithyroid, anticholinesterase and curarising agents. In toxicology, application of an analogous principle has produced dimercaprol. The principle which has already been so successful in the above work is already being applied in cancer research, using the experience gained in these fields. It is perhaps a relief to realise that other types of work have also borne important fruit, such as pteroylglutamic acid, vitamin B₁₂ and dextran.

Two editions of Sollman's Manual of Pharmacology have appeared during this remarkable decade. The edition of 1942 has already established itself, like its predecessors, as a book in which a busy doctor, teacher or research worker may find either the pharmacological information he requires, or references to original papers in which to search further. The clear arrangement of this book, its concentration on what is important and its comprehensive system of references make it extremely useful as a source of information. The question naturally arises as to whether the seventh edition is as successful as its predecessors.

Since the layout of the seventh edition closely follows that of the sixth, the question resolves itself into one of how far the new edition includes and assimilates the developments of the intervening years. It is astonishing to find, owing to the fast growth of pharmacology, that many of the substances now occupying the minds of research workers and clinicians, such as aureomycin, chloromycetin and polymyxin among antibiotics, vitamin B₁₂ and its associated factors, C10 and antrycide, are necessarily omitted, owing to their discovery or development having come too late for inclusion in this edition. Researches published slightly earlier are, on the other hand, treated in the excellent way we have come to expect from Professor Sollman.

For the English reader it is worth mentioning that, as is perhaps natural in an American textbook, attention tends to be concentrated on drugs likely to be of interest to the American reader. For example, chloroquine is given fifteen times as much space as proguanil (paludrine). Moreover, the reader is generally referred to the American literature, which may be more accessible in that continent.

H. O. J. COLLIER.

NEW APPARATUS

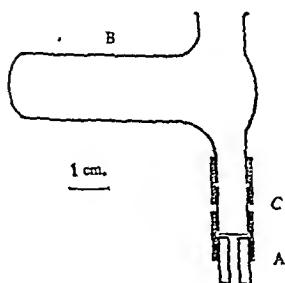
AN APPARATUS FOR SEMI-MICRO CRYSTALLISATION

BY W. C. EVANS AND M. W. PARTRIDGE

From the University of Nottingham

Received March 16, 1949

REPEATED recrystallisation of quantities of material of the order of 20 to 40 mg. often presents a difficult problem. Application of the usual micro-methods or of the conventional macro-methods, using small apparatus, frequently results in disappointing losses of material owing to premature crystallisation during filtration or to repeated transferences of small quantities of solutions or solids from vessel to vessel. We have found that an apparatus, consisting essentially of a crystallisation vessel attached to a form of the Schwinger filter, is very convenient for repeated and rapid crystallisations. The principle of the apparatus is somewhat similar to that of the Bergkamp¹ filter-beaker.



The apparatus, having the dimensions shown in the figure, is convenient for crystallisations from about 0.4 to 4 ml. of solvent. The sur-

faces, which meet inside the rubber sleeves, are ground flat. Cell C is tared together with the filtering unit A, and the disc of hardened filter paper secured by the lower rubber sleeve. The crude reaction product is collected, washed and dried in cell C, slight positive pressure being applied to B for rapid filtration. After weighing the product, the cell C and filtering unit are again attached to the crystallising vessel B and the thick-walled capillary tube is replaced by a plug of glass rod of the same dimensions. Most of the solid is tapped down into B and dissolved in hot solvent; by tilting the apparatus, the hot solution is run to and fro into C to dissolve solid adhering to the sides. Cell C is then removed and the filtering unit is attached directly to the crystallising vessel. The solution is reheated for a short time to warm the whole apparatus and then filtered, under slight positive pressure, into a micro-beaker by turning the apparatus through 90°. If crystallisation occurs during filtration, the crystals are easily dissolved in more hot solvent run to and fro from B and then filtered into the bulk of the solution.

The crystals which separate are collected in the same manner as the crude reaction product. Since filtration of the hot solution in further crystallisations is not normally necessary, the crystals are transferred to B as before and dissolved; crystallisation is then allowed to take place in B. The crystals are collected again in cell C. If some crystals adhere to the sides of B, they are redissolved in some of the mother liquor, allowed to crystallise and collected with the main bulk in cell C. The crystals are then washed and dried.

The process can be repeated as often as necessary. After the first hot filtration, there are no losses owing to transference from vessel to vessel, and the material is accessible for weighing and melting-point determination between each recrystallisation.

In the first two trials with this apparatus, hyoscyamine picrate, obtained

LETTERS TO THE EDITOR

Aluminium Oxide for Quantitative Chromatographic Analysis.

SIR,—In my article on *The British Pharmacopœia*, 1948, *The Assay of Alkaloidal Salts*, which appeared in No. 1 of your Journal, I stated that aluminium oxide for quantitative chromatographic analysis must be completely free from alkali and must give a good adsorption test. I wish now to add a more complete set of tests which I would recommend if the chromatographic method is adopted.

Aluminium oxide for quantitative chromatographic analysis should comply with the following tests:—

Neutrality: Shake 1 g. of aluminium oxide with 20 ml. of water and filter; the filtrate is neutral to litmus paper.

Adsorption: Weigh 5 g. into an Erlenmeyer flask, add 20 ml. of a 1 per cent. procaine hydrochloride solution in alcohol and set aside for 15 minutes occasionally swinging the flask. Filter through a filter of diameter 11 cm. To 10 ml. of the filtrate add 10 ml. of water and 5 drops of bromothymol blue solution and titrate to a green colour with 0.1 N hydrochloric acid; 1.90 to 2.40 ml. should be required.

Volume. Pour 10 g. into the glass tube used for the determination, keeping the tube vertical against a firm surface and allowing the aluminium oxide to fall in ten portions from a height of 1 cm. The height of the column in the glass tube should then be 12.5 to 14.5 cm. Attach the glass tube with the aluminium oxide to a suction flask and draw 10 ml. of alcohol (90 per cent.) at 390 to 410 mm. Hg. through the column. The time from the beginning of the suction until the last of the alcohol has been drawn through the column should be $2\frac{1}{2}$ to 5 minutes. A further 30 ml. of alcohol (90 per cent.) is then passed through the column in the same manner; the filtrate thus obtained is used for the following tests.

Alkalinity and Acidity. To 10 ml. of the filtrate add 10 ml. of water and 5 drops of bromophenol blue solution, a yellow or green colour is produced.

Soluble Substances. Evaporate 10 ml. of the filtrate to dryness and dry at 105°C. to constant weight. The weight of the residue must not exceed 2 mg.

Department of Pharmaceutical Chemistry,

D. VAN OS.

The University,

Groningen, Netherlands.

NEW REMEDIES (continued from page 349)

preparations, only the maintenance dose should be used. It is supplied in packages of 30, 100 and 500 tablets containing either 0.1 or 0.2 mg. S. L. W.

Estigyn* is 17-ethinyl œstradiol, an ethinyl derivative of the naturally-occurring œstrogenic steroid α -œstradiol. Weight for weight it is claimed to be the most active œstrogenic substance known and is active when given orally. It is non-toxic in the usual therapeutic doses and is well tolerated. It is indicated in all conditions calling for treatment with œstrogens, especially hypo-ovarianism, menopausal disorders, inhibition of lactation and prostatic carcinoma. The usual dose is 0.05 mg. 3 times daily, though this may be increased to 6 times daily for inhibition of lactation. It is supplied in bottles of 25, 100 and 500 tablets each containing 0.05 mg. S. L. W.

Ethiodan* is ethyl-*p*-iodophenylundecate, a mobile liquid used as a contrast medium for myelography, sp. gr 1.264 at 20°C., iodine in organic combination approximately 30 per cent. It is more stable than iodised oil and

[Continued on page 352]

NEW APPARATUS

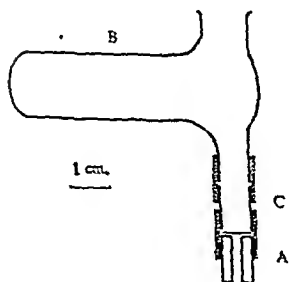
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Received March 16, 1949

REPEATED recrystallisation of quantities of material of the order of 20 to 40 mg. often presents a difficult problem. Application of the usual micro-methods or of the conventional macro-methods, using small apparatus, frequently results in disappointing losses of material owing to premature crystallisation during filtration or to repeated transferences of small quantities of solutions or solids from vessel to vessel. We have found that an apparatus, consisting essentially of a crystallisation vessel attached to a form of the Schwinger filter, is very convenient for repeated and rapid crystallisations. The principle of the apparatus is somewhat similar to that of the Bergkamp¹ filter-beaker.



The apparatus, having the dimensions shown in the figure, is convenient for crystallisations from about 0.4 to 4 ml. of solvent. The surfaces, which meet inside the rubber sleeves, are ground flat. Cell C is tared together with the filtering unit A, and the disc of hardened filter paper secured by the lower rubber sleeve. The crude reaction product is collected, washed and dried in cell C, slight positive pressure being applied to B for rapid filtration. After weighing the product, the cell C and filtering unit are again attached to the crystallising vessel B and the thick-walled capillary tube is replaced by a plug of glass rod of the same dimensions. Most of the solid is tapped down into B and dissolved in hot solvent; by tilting the apparatus, the hot solution is run to and fro into C to dissolve solid adhering to the sides. Cell C is then removed and the filtering unit is attached directly to the crystallising vessel. The solution is reheated for a short time to warm the whole apparatus and then filtered, under slight positive pressure, into a micro-beaker by turning the apparatus through 90°. If crystallisation occurs during filtration, the crystals are easily dissolved in more hot solvent run to and fro from B and then filtered into the bulk of the solution.

The crystals which separate are collected in the same manner as the crude reaction product. Since filtration of the hot solution in further crystallisations is not normally necessary, the crystals are transferred to B as before and dissolved; crystallisation is then allowed to take place in B. The crystals are collected again in cell C. If some crystals adhere to the sides of B, they are redissolved in some of the mother liquor, allowed to crystallise and collected with the main bulk in cell C. The crystals are then washed and dried.

The process can be repeated as often as necessary. After the first hot filtration, there are no losses owing to transference from vessel to vessel, and the material is accessible for weighing and melting-point determination between each recrystallisation.

In the first two trials with this apparatus, hyoscyamine picrate, obtained

from hyoscyamine sulphate solution produced in a partition chromatogram of *Atropa Belladonna*² was used. The results of crystallising two samples of the picrate from aqueous alcohol are shown in the Table. We have since obtained equally satisfactory results in many other crystallisations.

Recrystallisations	Weight recovered mg.		Melting-point °C. (uncorrected)	
	(a)	(b)	(a)	(b)
Crude precipitate	21	40	162—3	162—3
First crystallisation with filtration of hot solution ...	14.5	34	164—5	164—5
Second crystallisation without filtration of hot solution	11.5	32	164—5	164—5
Third crystallisation without filtration of hot solution ...	10.5	30	164—5	164—5
Fourth crystallisation without filtration of hot solution	9.5	27	164—5	164—5

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1. von Bergkampff, *Z. anal. Chem.*, 1926, 69, 321.
2. Evans and Partridge, *Quart. J. Pharm. Pharmacol.*, 1948, 21, 126.

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is less irritating; its low viscosity also makes it easier to handle, and a comparatively small gauge needle may be used. Ethiodan is specifically indicated for use in the radiological diagnosis and localisation of cord tumours, herniated nucleus pulposus, intraspinal protrusion of intervertebral discs, and any other conditions in which obstructions in the cerebrospinal canal or compression of the cord are suspected. Normally, 3 ml. is injected immediately below the level at which the obstruction is suspected. It is issued in boxes of 3 ampoules each containing 3 ml.

S. L. W.

Ferosan Tablets* contain exsiccated ferrous sulphate 3 gr., copper sulphate 1/25 gr., and manganese sulphate 1/25 gr. Their use is indicated in all cases of hypochromic microcytic anæmia, including anæmia due to chronic or acute hæmorrhage, idiopathic hypochromic anæmia and anæmia of pregnancy or lactation. The adult dosage is 1 or 2 tablets 3 times daily after meals. Ferosan tablets are supplied in bottles of 100 tablets.

S. L. W.

Priscol* is the hydrochloride of 2-benzyl-4:5-imidazoline; in colourless crystals, freely soluble in water; m.pt. 171°C. Its principal action is to dilate the peripheral vessels. This effect is primarily on the arterioles and the smaller arteries, and its use is therefore followed by hyperæmia and acceleration of the blood flow in the capillaries. The improved circulation is usually accompanied by a fall in blood pressure. It is indicated particularly for the treatment of peripheral vascular disorders, by intravenous, intramuscular or intra-arterial injection; arthritic conditions are treated by peri-articular injections or the local use of an ointment. It may also be employed as a local application, combined with parenteral or oral therapy, for the treatment of slow-healing wounds and ulcers. It is claimed to be especially valuable in ophthalmic conditions where active hyperæmia is desired; for this purpose, it is employed either in the form of drops of a 10 per cent. solution or by subconjunctival injection. Priscol is supplied in bottles of 40 or 200 tablets containing 25 mg., in boxes of 10 ampoules containing 1 ml. (10 mg.), in bottles of 10 ml. of 10 per cent. solution, and in tubes containing 20 g. of 10 per cent. ointment.

S. L. W.

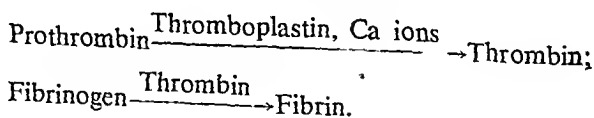
REVIEW ARTICLE

ANTICOAGULANTS

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THE processes that underly the clotting of blood are still far from adequately understood, in spite of the many facts uncovered by generations of patient investigators. The classical scheme associated with the name of Morawitz¹, although neither complete nor universally accepted, is still useful as a summary of the main events. According to this scheme, the plasma contains three essential ingredients of the complete clotting system: these are ionised calcium and the proteins prothrombin and fibrinogen. The addition of a fourth ingredient, thrombokinase (or better thromboplastin, since its enzymic nature is still in doubt) completes the system, and initiates clotting. Thromboplastin is present in the tissues generally and also in the blood platelets. When blood makes contact with tissue fluid, or the platelets are injured by contact with a hydrophilic surface, prothrombin reacts with thromboplastin and with calcium ions to form an enzyme, thrombin: this is the first stage of clotting. In the second stage of clotting thrombin acts upon fibrinogen, changing it into the insoluble protein fibrin, whose threads constitute the matrix of the clot. Or in summary:



Any substance, or treatment, which removes or inactivates any of the five clotting factors will prevent coagulation. Anticoagulants might therefore be divided into five groups, according to the factor interfered with: such a classification, however, would not be a very useful one, since some important anticoagulants affect more than one component of the system, and since a single component may be attacked by anticoagulants differing completely in their mode of action. In addition, it must be emphasised that the Morawitz scheme is oversimplified. Thus, at least one further plasma protein^{2,3,4} is involved in the conversion of prothrombin to thrombin; and prothrombin itself has been regarded as a complex of two easily separable factors^{5,6,7}. The velocity of both stages of clotting is related to the nature and concentration of the electrolytes present, and that of the second stage is reduced by antithrombin, of which traces are always present in plasma and further amounts are set free during clotting. Finally, the relation of the plasma proteases and their inhibitors to clotting is still poorly defined. No further mention will be made of these factors, and the action of anticoagulant substances will be discussed in terms of the Morawitz scheme alone.

MODES OF ACTION OF ANTICOAGULANTS

A list of the possible mechanisms of anticoagulant action is given below, with a number of the most important substances exhibiting each type of activity.

Prevention of platelet disintegration: hydrophobic surfaces (paraffin, amber, perspex, collodion, silicone, etc.); most anticoagulants.

Removal of calcium ions: oxalates, fluorides, citrates, soaps of alkali metals, ion-exchange resins.

Interference with prothrombin formation: dicoumarol, salicylates.

Inhibition of the conversion of prothrombin to thrombin: heparin, other sulphuric acid esters, salts of the rare earth metals, organic bases, reducing agents, trypsin inhibitors.

Inhibition of the action of thrombin on fibrinogen: heparin, other sulphuric acid esters, reducing agents (cysteine, glutathione, bisulphite, etc.), 'lipid inhibitors', organic bases, trypsin inhibitors.

Inactivation of fibrinogen: fibrinolysin, protamines.

Release of heparin from the tissues: peptone, antigens (in sensitised animals), radioactive substances, nitrogen mustards, diamines, diamidines, etc.

The following discussion will be concerned chiefly with those substances that are now used with the primary aim of inhibiting coagulation *in vitro* or *in vivo*. Only brief mention will be made of substances formerly so used, but now discarded in favour of more active or less toxic materials, and of substances whose anticoagulant action is important only as a side-effect of their therapeutic employment.

SUBSTANCES PREVENTING THE DISINTEGRATION OF THE PLATELETS

It is difficult to withdraw blood from a vein or artery without contaminating it with tissue fluid, but with good technique (clean puncture, avoidance of stasis, discarding of the first portion of effluent blood) the contamination may be kept small. The speed of clotting then depends, other factors being equal, on the rate of platelet disintegration, and this in turn depends on the surface with which the blood is in contact. Clotting is promoted by increasing the area of contact; the large contact area is the main factor in the hæmostatic efficiency of gauze and of its absorbable substitutes such as fibrin foam and gelatin foam. The chemical nature of the surface is equally important. Clotting is slower in a Pyrex glass vessel than in a soda-glass vessel, and still slower in a vessel lined with a water-repellant substance such as paraffin, amber, collodion or any of a variety of plastics. The best of all surfaces for the delay of platelet lysis is provided by the silicone film formed by the hydrolysis of dimethyldichlorosilane³. Blood taken with silicone-coated syringes and needles, and kept in silicone-coated vessels, may remain fluid for several hours. The silicone technique should facilitate the study *in vitro*, or in perfusion experiments of phenomena depending on the presence of normally reactive platelets: for example, the liver of a sensitised dog may be perfused with normal whole blood, and can then respond with a maximum anaphylactic reaction when the specific antigen

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is added to the perfusion stream, an effect not obtained when heparinised or defibrinated blood is used for the perfusion⁹.

The mechanism of platelet lysis by contact with foreign surfaces is little understood: there is some evidence¹⁰ that a plasma factor takes an active part. Anticoagulants generally, and notably heparin, delay the lysis, perhaps through an action on the plasma factor rather than directly on the platelets themselves. *In vivo*, platelets agglutinate on a damaged area of endothelium; *i.e.* they stick to one another as well as to the injured blood vessel wall, forming a "white thrombus" or "platelet clot." When the clumped platelets lyse, fibrin is formed locally; in addition, the clumping is itself in some way favoured by the processes giving rise to the fibrin clot. Wright¹¹, applying a simple quantitative test for the measurement of platelet "stickiness," found that this was reduced in animals treated with anticoagulant drugs, including dicoumarol¹², which has no important direct effect on any component of the clotting system, but owes its activity entirely to its ability to prevent prothrombin formation. A platelet thrombus may form within a vessel even when fibrin formation is completely inhibited, as for instance by the administration of heparin; if the dose of heparin is pushed still higher, platelet agglutination is stopped too. The effect of heparin on platelet agglutination begins later and lasts longer than that on the clotting time¹³. These observations are among many which emphasise the dissociability of platelet agglutination and clotting in the ordinary sense; yet the two processes are favoured or prevented by various common influences, and their interrelationships are hard to disentangle, except for the obvious fact that the platelet thrombus is a rich potential source of thromboplastin, and to that extent a likely site of fibrin formation. Further study is needed of the plasma factors affecting platelet adhesiveness and fragility. The observation of Wright¹⁴ that the platelets become more sticky after surgical operations may perhaps be related to the finding by Maofarlane and Biggs¹⁵ of increased fibrinolytic activity at this time, as well as to the greatly increased risk of intravascular clot formation.

DECALCIFYING ANTICOAGULANTS

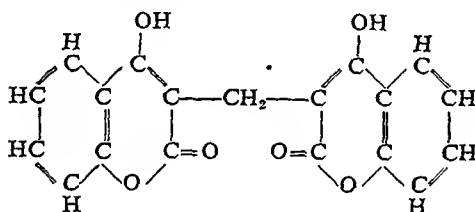
It has been known for over fifty years that calcium precipitants like oxalates, fluorides and the soaps of alkali metals can inhibit the clotting of shed blood, and that the inhibition can be removed by adding an excess of soluble calcium salt. Citrates act similarly, but without precipitating calcium, which becomes bound as part of a complex anion¹⁶. The cheapness and low toxicity of citrate have made it the anticoagulant of choice in blood transfusion. The injected citrate is so greatly diluted by the body fluids that it has no anticoagulant action *in vivo*: the clotting time may even be shortened somewhat¹⁷. When very large volumes of blood or plasma have to be transfused within a short time, the toxicity of citrate may become significant.¹⁸

The prevention of clotting in blood treated with ion-exchange resins, through replacement of the plasma Ca by Na, has recently been described¹⁹.

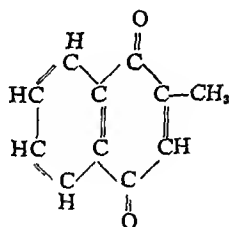
SUBSTANCES INTERFERING WITH PROTHROMBIN FORMATION

It has long been believed that prothrombin is manufactured by the liver, and this belief has been fully substantiated by recent investigations. When the liver is extirpated or severely damaged (e.g. by carbon tetrachloride) the plasma prothrombin falls within a few days to a negligibly low level. Less extensive liver injury produces a smaller, but still readily detectable, reduction in plasma prothrombin. The formation of prothrombin can, however, be depressed without noticeably interfering with the other functions of the liver. The substance which most clearly acts in this way is dicoumarol.

Dicoumarol. The observations of Schofield²⁰ and Roderick²¹ showed that the hæmorrhagic condition of cattle fed on spoiled sweet-clover hay was due to the ingestion of a water-soluble toxic principle, and made it probable that prothrombin was the point of attack. The remarkable work of Link and his colleagues^{22,23,24} led to the identification of the hæmorrhagic agent as 3,3'-methylene-bis-hydroxycoumarin, now known as dicoumarol.



Dicoumarol

2-methyl-1:4-naphthoquinone
(Menaphthone)

There is no doubt that the whole of the anticoagulant action of dicoumarol is due to its interference with prothrombin synthesis: it has practically no effect on clotting *in vitro*. The structural similarity of the dicoumarol half-molecule to the compounds of the vitamin K group (menaphthone is a synthetic vitamin-K analogue: the natural vitamins have branched unsaturated alkyl chains instead of methyl in the 2-position) early suggested that dicoumarol acted as an antagonist to the vitamin. Although the first attempts to counteract the action of dicoumarol by treatment with menaphthone were unsuccessful, it is now clear that the two compounds act antagonistically over a certain range: the vitamin must, however, be given in doses far exceeding the usual therapeutic ones. Ascorbic acid potentiates the anti-dicoumarol effect of menaphthone, and it has been suggested²⁵ that the bleeding tendency characteristic of chronic dicoumarol poisoning may be due in part to a disturbed vitamin C metabolism. Further evidence of the relation of dicoumarol to vitamin K comes from the observations of Meunier and his colleagues^{26,27}, who have described coumarin derivatives with vitamin K activity as well as naphthoquinones acting like dicoumarol. The view that dicoumarol acts by blocking the vitamin is now generally accepted,

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but its mode of action cannot be further analysed, since the role of the vitamin itself in prothrombin formation is unknown.

Dicoumarol is a weak acid, nearly insoluble in water but forming water-soluble salts. Unlike most other anticoagulants, it is active by mouth. The clotting time as ordinarily measured does not provide a satisfactory index of the effectiveness of dicoumarol, and some form of prothrombin-time test is universally used in both experimental and clinical work to gauge the effectiveness of the drug. The action on prothrombin-time is a delayed one, since time must be allowed for the existing prothrombin to disappear: the maximum prolongation is seen in 2 to 4 days after the administration of a single dose. A rather longer time is required for the restoration of prothrombin after the effect has begun to wear off. In the presence of liver injury the effect of the drug is enhanced both in degree and in duration; the same is true when the kidney is damaged. There is no doubt that the response to dicoumarol varies considerably from subject to subject: the effect of an excessive reduction of prothrombin can be counteracted by the administration of a vitamin K preparation, or (since symptoms do not occur unless the plasma prothrombin is depleted to a small fraction of the normal value) by the transfusion of normal blood or plasma.

The toxicity of dicoumarol for both animals and man is related almost entirely to its anticoagulant action, and death when it occurs is due to hæmorrhage. Morphological changes in the liver, when seen at all, are usually secondary to local hæmorrhage, and most liver function tests reveal no impairment of the organ. The plasma fibrinogen level is, however, somewhat raised by moderate doses of dicoumarol, and reduced by large doses²⁵: similar effects are produced by various agents toxic to the liver, and it may be that the abnormality in this organ is not strictly confined to the prothrombin-forming system. Plasma fibrinogen levels are, however, distinctly labile, and the degree of general hepatic injury produced by dicoumarol is at most an extremely slight one.

Salicylates. Link²⁴ has suggested that the action of dicoumarol may be an indirect one: its breakdown within the body may liberate salicylates, and these may be responsible for inhibiting the formation of prothrombin. While there is not yet enough evidence to prove or disprove this idea, the deleterious action of salicylates on prothrombin formation has been amply confirmed in both human and animal studies.

SUBSTANCES INHIBITING THE CONVERSION OF PROTHROMBIN TO THROMBIN AND/OR THE ACTION OF THROMBIN

It might be thought convenient to consider in separate sections the substances opposing the formation of thrombin, and the substances impeding its action. In practice this is difficult. Heparin, the most important of all, acts on both stages of clotting; and the same is true of some, at least, of the anticoagulants which resemble it in being sulphuric acid esters of high molecular weight. Even in the case of heparin it is very hard to assess the relative contribution made by its antiprothrombin and antithrombin activities to its overall effect on clotting systems containing

whole blood; and the same statement applies with greater force to the related anticoagulants, none of which has been investigated in such detail as heparin. Antiprothrombin activity, in turn, might be due to inactivation of either thromboplastin or prothrombin itself. In practice, the amount of anticoagulant required to prevent thrombin formation goes up if the clotting system is enriched in either thromboplastin or prothrombin. This, however, would be expected on the basis of any theory postulating a reversible combination between either of the clotting factors and the inhibitor. It is likely that both prothrombin and thromboplastin may combine with the anticoagulant and lose activity on so doing; but until the factors concerned in thrombin formation can all be isolated and studied by adequate physio-chemical methods, the relative importance of the various possible reactions can only be guessed at.

Most of the anticoagulants listed as possessing antiprothrombin or antithrombin activity have large molecules, or can form more or less stable complexes with protein constituents of the plasma. They thus remain in the blood for some time after injection, and their anticoagulant action, unlike that of the decalcifying anions, is demonstrable *in vivo* as well as *in vitro*. Many of them are compounds containing sulphuric acid in ester linkage. Of these heparin has received by far the most attention, both because it is a natural constituent of the body and may be concerned in maintaining the normal fluidity of the blood, and because its toxicity is low enough to permit its prolonged administration to patients in danger from intravascular thrombosis.

Heparin. The monograph by Jorpes³² is a recent and comprehensive treatise on the chemistry, physiological action and clinical applications of the substance. Earlier summaries by Best^{33,34} and Wilander³⁵, both of whom have made important contributions in this field, are still well worth consulting.

History and chemistry of heparin. Heparin was discovered by McLean³⁶ in 1916 during an investigation, under Howell's direction, of the thromboplastic action of phosphatide preparations from liver and heart. During the next ten years Howell and his colleagues studied it intensively and showed that it was not, as had been thought at first, a lipid; their best preparations contained uronic acids and had a high ash content. In the further purification of heparin and in the elucidation of its chemical nature, the major part was played by Charles and Scott in Toronto and by Jorpes and his co-workers in Stockholm. The Canadian workers³⁷ devised an alkaline extraction technique which gave an improved yield of a purer product, and they showed that many mammalian tissues contained heparin, ox lung being a particularly rich source. The Swedish investigators^{38,39} confirmed the presence of a uronic acid in the purified material, and identified glucosamine and ester sulphate as further constituents: the remarkably high content of sulphate explained the large proportion of ash in Howell's material. Jorpes and Bergstrom³⁰ concluded that heparin is a mucoitin polysulphuric acid, and this view has been sustained by subsequent investigation.

About the same time Charles and Scott⁴⁰, who had been continuing

their purification studies, reported the isolation of a crystalline barium salt of heparin having a constant composition. There has been a good deal of controversy about the significance of this material. Its isolation has been repeated and the formula $C_{28}H_{44}O_{55}N_2S_5$ has been suggested^{41,42} for the corresponding heparin acid, but Jorpes³² regards the barium salt as a mixture of compounds varying in their degree of esterification. There is little doubt that the barium salt of Charles and Scott was really crystalline and that the crystallisation procedure is a valuable method of purification. The heparin molecule is, however, such a large one (the molecular weight of the barium salt is 3462 according to the formula of Charles and Todd and the compound may be a polymer) that crystallinity alone is an insufficient guarantee of purity. There is, indeed, a good deal of evidence that the crystallised preparation does not necessarily represent a chemical individual; samples of it vary appreciably in composition³² and have been separated into fractions of unequal potency⁴³; the potency may go down with repeated recrystallisation⁴⁴; and the barium salts obtained from different mammalian species are of quite divergent potency, although similar in elementary composition, crystalline form, and chemical behaviour⁴⁵. It is hard to say whether heparin in its natural state ought to be regarded as a chemical individual, difficult to obtain in strictly unmodified condition, or whether a family of closely related heparins exists in the tissues: the latter is perhaps the more likely alternative, if only because so complex a substance is probably not synthesised in a single step. Nevertheless, it seems probable that the best preparations obtained by the Canadian and the Swedish workers, and by others, represent a near approach to the most potent heparin obtainable, at any rate from bovine tissue.

The standardisation of heparin. The difficulty of obtaining heparin of uniform quality makes it desirable that each preparation should be standardised for potency. Howell⁴⁶ originally defined a unit of heparin activity as the minimum amount which, when added to 1 c.c. of freshly drawn cat's blood, would keep it fluid for 24 hours. Experience with many drugs has shown that such a unit, defined in terms of a poorly reproducible biological system, cannot be relied on to be constant; and in accordance with the practice now generally accepted, several groups of workers set up stable reference preparations, by comparison with which the potency of other samples was determined. These have now been replaced by an International Standard Heparin⁴⁷; the bulk of which is preserved at the National Institute for Medical Research, London, N.W.3, under the auspices of the Committee on Biological Standardisation of the World Health Organisation. The International Unit represents the strength of 1/130 mg. of this preparation: it is practically identical with the Toronto unit⁴⁸, defined in terms of a sample of barium salt, and, so far as can be ascertained, roughly equal to the Howell unit. It is to be expected that the practice of labelling potency in units will become superfluous in time, because all commercial preparations will be of the same maximum strength: but meanwhile the common practice of reporting doses in mg. introduces an uncertainty of at least 30 to 40 per cent.

and is to be deprecated. It is perhaps unfortunate, as Jorpes³² points out, that the established unit is such a small one: it is inconvenient to have to write out "30,000 units", for instance, when referring to a single human dose.

A great variety of assay methods has been suggested for the standardisation of heparin preparations: the clotting systems which have been used include fresh blood; citrated or oxalated blood or plasma, recalcified with or without the addition of tissue extract; fresh fowl plasma plus tissue extract; and whole blood or plasma plus thrombin. Reproducible results can be obtained with any of these systems, but when two samples of heparin are compared by different methods the values obtained for the relative potency may differ somewhat: this is particularly true when the samples are obtained from different species⁴⁹ or when one of them has been denatured by treatment with acid⁵⁰. The cause of such discrepancies is presumably the varying affinity of the several active principles for different constituents of the clotting mixture. As it appears that most of the heparin now on the market is of bovine origin, and is at least two-thirds as active as the best preparation obtainable from the species, the uncertainties due to the use of varying assay methods are not very serious.

Physical and chemical properties of heparin. Heparin and its salts with the alkali and alkaline earth metals are colourless substances, soluble in water but not in most organic solvents; they do not dialyse appreciably through ordinary collodion membranes. The sodium salt, which is the form usually supplied, is very stable in neutral or alkaline solution and can be sterilised by heating; when heated in acid solution it slowly loses its activity. Heparin is precipitated by many organic bases, including benzidine, protamines, toluidine blue and streptomycin: this property has been employed for inactivating heparin both *in vitro* and *in vivo*. Toluidine blue changes when combined with heparin into its reddish-violet "metachromatic" tautomer⁵¹; a similar sort of colour change is shown by other basic dyes in the presence of heparin. The metachromatic reaction was shown by Lison⁵² to be specific for sulphuric acid esters of high molecular weight: it is given by a large number of substances, both natural and synthetic, which possess anticoagulant activity. The Swedish workers, in a series of particularly elegant experiments^{53,54,55}, have shown that the granular material of the mast cells, which takes an intense metachromatic stain, is rich in heparin.

The most striking property of heparin is the remarkably strong negative charge carried by its molecule in aqueous solution. Indeed, as Jorpes points out, this is almost its only important chemical property, since apart from its acidic (sulphuric and carboxyl) radicals the molecule has no reactive groups. The acidic strength of heparin enables it to react with the basic groups of proteins and other substances and is certainly the basis of its anticoagulant action. That its affinity for proteins is a general one and is not confined to the proteins concerned with clotting was shown by Fischer⁵⁵, who observed that the isoelectric point of casein was shifted to the acid side in the presence of heparin.

Mode of action of heparin. Although there is no doubt that the negative charge carried by its molecule is responsible for the characteristic activity of heparin, the activity is not simply a function of the proportion of esterified sulphuric acid that is present. The large size of the molecule is also involved in some way. The low potency of the heparin obtained from some mammalian species, and the reduced activity of heparin subjected to mild treatment with acid, which have been referred to, probably indicate a correlation between potency and degree of polymerisation⁴⁴. That potency increases with molecular size is more obvious in the case of the synthetic esters of sulphuric acid, which will be referred to later.

While the key to the action of heparin must be sought in the large size and electronegativity of its molecule, these properties do not explain why heparin should act particularly on the blood-clotting system. Indeed, it is by no means certain that its physiological function has to do with the maintenance of the fluidity of the blood. Heparin does, in fact, act on other enzyme systems: it neutralises complement⁵⁶; antagonises fumarase⁵⁷, trypsin⁵⁸, and fibrinolysin⁵⁹; and it has some inhibitory effect on a variety of allergic reactions. When it is injected or liberated into the blood stream, its low diffusibility will tend to keep it there, and it may be in part for this reason that it has so few extravascular actions.

Heparin can prevent both the conversion of prothrombin to thrombin and the clotting of fibrinogen by thrombin. In either case the inhibitory action disappears when the isolated clotting reagents are used. Thus, heparin does not inhibit the clotting of purified fibrinogen by purified thrombin⁶⁰, or the formation of thrombin from purified prothrombin⁶¹. An additional factor, heparin complement^{62,63,64}, must be present if either reaction is to be prevented: this substance is a constituent of the albumin fraction of the plasma, but is not the crystallisable serum albumin proper. The antithrombin of normal plasma is also found in the albumin fraction, and the attractive suggestion has been made that it may be heparin-complement combined with a small fraction of its possible charge of heparin. The balance of evidence^{64,65} seems at present, however, not to support this hypothesis.

Heparin: fate in the body. Heparin given by vein exhibits its greatest effect on clotting within the first few minutes. It is then rather rapidly inactivated or removed from the circulation. Some is excreted in the urine^{66,67,68}; the greater part apparently escapes slowly into the tissues, where it may be enzymatically inactivated⁶⁹. The duration of the anticoagulant effect is roughly proportional to the dose, but does not exceed 2 to 3 hours unless the dose is so great that its initial effect is to raise the clotting time to infinity.

Organic esters of sulphuric acid. The possibility of obtaining a cheap, synthetic anticoagulant suitable for use *in vivo* began to receive attention about 20 years ago. The anticoagulant action of the trypanocidal drug suramin (sodium *m*-benzoyl-*m*-amino-*p*-methylbenzoyl-*l*-aminonaphthalene-4:6:8-trisulphonate, germanin, Bayer 205) had been known for some time⁷⁰, and in 1930 Stuber and Lang⁷⁰ reported its successful use

in the therapy of thrombosis. In the same year Rous, Gilding and Smith⁷¹ noted the anticoagulant effect of the dye chiazol blue 6B (chlorazol sky-blue FF, the sodium salt of tetrazotised dianisidine coupled with 1-amino-8-naphthol-2:4-disulphonic acid); and Demole and Reinert⁷² prepared a variety of substances of high molecular weight and found some of them active against clotting, the most potent being sulphonated aromatic polymers. One of these, the sodium salt of polyanetholesulphonic acid, was later marketed as "liquoid," and has had some popularity as an anticoagulant for *in vitro* and animal experiments: it is a rather toxic substance causing delayed death in doses not far above the effective ones. Chlorazol sky-blue, however, and some related azo-sulphonic dyes, especially Chlorazol fast-pink BKS (sodium 3:5-disulpho-diphenylurea-4:4-diazo-*bis*-2-amino-8-naphthol-6-sulphonate) were found by Huggett and his colleagues^{73,74} to be relatively non-toxic, and have been used extensively in physiological experiments. They act, apparently, mainly in the first stage of clotting. In this group of dyes there is no simple relationship between chemical structure and anticoagulant activity: all have heavy molecules containing a number of $-SO_3$ groups and azo linkages; and it is of interest that they resemble heparin, and the other synthetic anticoagulants containing ester sulphate, in having a strong affinity for toluidine blue and other metachromatic dyes.

The next series of synthetic anticoagulants to be investigated represented, chemically at least a nearer approach to heparin. Chargaff and his colleagues⁷⁵ and Jorpes' collaborator Bergstrom⁷⁶ independently reported the activity of carbohydrates esterified with sulphuric acid by treatment with chlorosulphonic acid. The esters derived from mono- and di-saccharides were inactive, but all the polysaccharides tested gave rise to active compounds. The treatment with chlorosulphonic acid, if unduly prolonged, yielded less active products, presumably because the materials were being depolymerized⁷⁶. These compounds probably act on both stages of clotting, and the best of them^{77,78,79} approach heparin in potency: the ratio of their activity to that of heparin cannot be exactly stated, since it varies greatly with the conditions of assay. All are too toxic for clinical use. It is of interest that their toxicity is due, at least in part, to their effect on clotting factors: they either cause the platelets to agglutinate, or precipitate fibrinogen, or both. According to Karrer and his colleagues⁸⁰ the toxicity is reduced by the introduction of other acid groups into the polysaccharide molecule before the treatment with chlorosulphonic acid.

Many high-molecular-weight esters of sulphuric acid occur naturally in both animals and plants; some of these are anticoagulants and some are not: activity is correlated at least roughly with ester sulphate content. Mucoitin and chondroitin sulphuric acids are not active, but become so when further esterified⁷⁶. The mucus of the mollusc *Charonia lampus* contains a potent anticoagulant⁸¹ with many chemical similarities to heparin. Two anticoagulants formerly popular with physiologists may possibly belong to this group, but little is known of their chemistry except that they seem to be acidic in nature: these are hirudin^{82,83}, from

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the buccal glands of the medicinal leech, and novirudin⁸⁴, a melanin-like substance of vegetable origin. A number of marine algae contain polysaccharides esterified with sulphuric acid. Agar-agar, the best known of these, is not an anticoagulant; but related substances from *Chondrus crispus*⁸⁵ and *Iridaea laminarioides*⁷⁵ are moderately active.

Basic dyes and other basic substances. All the substances just discussed presumably act in virtue of their strongly acid SO_3H groups and the size of their molecules. The possibility that basic substances of high molecular weight are also anticoagulants has been investigated. This too is the case: protamine and histones^{86,87} have well-marked activity, as have a number of basic dyes⁸⁷, including methylene blue, crystal violet and Janus green. Even so simple a base as diethylamine has some anticoagulant activity⁸⁸. The mode of action of these substances is supposed to be analogous to that of the synthetic heparin analogues, but in a reverse direction: i.e. they displace the isoelectric point of protein clotting-factors toward the alkaline side, and so reduce their reactivity. The antagonism between compounds of this group and the ester-sulphate anticoagulants has already been mentioned; it is due, however, not so much to a cancelling-out of opposed actions on the electric charge of protein clotting factors, as to a simple co-precipitation of the acidic and basic anticoagulants. None of the bases so far investigated appears likely to have any practical value in delaying coagulation.

Reducing substances. A number of reducing agents, both inorganic and organic, have anticoagulant activity; they appear to act on both stages of clotting. Sodium bisulphite⁸⁹ and thiosulphate⁹⁰, cysteine⁹¹ and glutathione⁹² may be mentioned.

Salts of rare earth metals. The trivalent cations of neodymium^{93,94} praseodymium, lanthanum and other elements of this group⁹⁵ are extremely active anticoagulants both *in vitro* and *in vivo*. They appear to act mainly on the first stage of clotting^{94,96}, but the mechanism of their action is not understood. The compounds are too toxic to be of practical value⁹⁷.

"Lipid inhibitors" of coagulation have been detected in phosphatide fractions of tissue extracts by Chargaff⁹⁸ and de Sütö-Nagy⁹⁹. Their mode of action has not been studied in detail, and whether they have any physiological significance for the prevention of clotting is unknown.

Trypsin inhibitors. The purified trypsin inhibitors obtainable from pancreas and from soya beans are fairly active in delaying coagulation^{100,101,102,103}. Their mode of action is obscure.

SUBSTANCES INACTIVATING FIBRINOGEN

The fibrinolytic enzyme of normal plasma, discovered forty years ago by Nolf¹⁰⁴, should be listed for the sake of completeness, since it can digest fibrinogen as well as fibrin¹⁰⁵. It is usually present in an inactive form, but can be activated *in vitro* or *in vivo* by a number of different ways. Whatever the relation of this enzyme to the clotting system, it is doubtful whether it ever, *in vivo*, destroys fibrinogen so rapidly as to make the blood incoagulable.

Fibrinogen is precipitated more or less selectively by both protamines and certain anticoagulants of the sulphonic ester group, but this is certainly not the main reason why such substances inhibit clotting.

SUBSTANCES CAUSING THE RELEASE OF HEPARIN

The blood of dogs thrown into shock by the injection of large amounts of Witte's peptone, or of an antigen (in previously sensitised animals), becomes incoagulable; and it has been conclusively demonstrated that the inhibition of clotting seen in such animals is due to circulating heparin^{107,108}. The liver is the principal source of the released heparin. A similar effect is produced by large doses of ionising radiation¹⁰⁹, by the radiomimetic drugs of the nitrogen mustard series¹¹⁰, and by certain simple basic drugs, particularly diamines and diamidines¹¹¹. Evidence that these stimuli release heparin in the same way from human tissues is lacking, except in the case of ionising radiations.

THE CLINICAL USE OF ANTICOAGULANTS

The utility of a non-toxic anticoagulant in the therapy and prophylaxis of thrombosis in man has long appeared probable, and was confirmed as soon as purified heparin became available in quantity. The first favourable clinical reports from Stockholm¹¹² and Toronto¹¹³ have been followed by some hundreds of papers describing the successful use of both heparin and dicoumarol in a variety of thrombo-embolic conditions. It is impossible to give a brief adequate summary of this work; an excellent account will be found in the monograph by Jorpes³², who seems, however, to emphasise unduly the toxic action of dicoumarol on the liver. Both drugs appear to have an established place in therapy. As compared with heparin, dicoumarol has the advantages of cheapness and of effectiveness on oral administration: its drawbacks are its slow onset of action, which makes it useless in emergencies unless supplemented by heparin, and the considerable variability in the response of individual patients. All anticoagulant therapy involves the risk of hæmorrhage, and this risk can only be minimised by close supervision of the patient and frequent checks of clotting time (in the case of heparin) or prothrombin time (in the case of dicoumarol).

The numerically most important field of usefulness for these drugs has been the prevention and treatment of post-operative thrombosis, particularly after pelvic operations. The incidence of this complication is notoriously variable, and the availability of an effective therapy should not distract attention from the importance of simpler measures, especially active and passive movement of the limbs. Treatment is usually begun on the second day, when the risk of bleeding at the site of operation is small, and continued till the patient is ambulant. Opinions vary as to whether anticoagulant therapy should be used routinely after pelvic and abdominal surgery or reserved until signs of clot formation appear. Early diagnosis of latent thrombosis is naturally of the greatest importance, and

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phlebography of the lower extremities¹¹⁴ and tests revealing hypercoagulability of the blood, such as the heparin tolerance test of de Takats¹¹⁵, have been found useful for this purpose.

Other forms of active venous thrombosis respond equally well to anticoagulant therapy, which undoubtedly reduces the incidence of embolic complications. Thrombosis of the mesenteric veins, the retinal veins and the cavernous sinus, have all been treated successfully, in addition to the more common condition in which the initial site of clot formation is one of the deep veins of the lower leg. The status of the anticoagulant drugs in the treatment of occlusive coronary artery disease is still uncertain. They are quite useless in subacute bacterial endocarditis. Overdosage with heparin is treated by withdrawal of the drug, when the blood regains its normal clotting power within a few hours, or in emergency by the intravenous injection of protamine, which has an instantaneous effect. Dicoumarol overdosage can be corrected by the administration of massive doses of vitamin K preparations, or more rapidly by the transfusion of fresh blood or plasma.

Finally it should be mentioned that heparin is a valuable adjunct to vascular surgery, and has some advantages over citrate as an anticoagulant in blood transfusion.

The expense and inconvenience of heparin therapy have undoubtedly restricted its field of usefulness. While intravenous administration, either several times a day or by continuous drip, is still the method most commonly used, a number of menstrua for the incorporation of heparin have been devised^{116,117}, which permit a prolonged effect to be obtained with a smaller number of intramuscular or subcutaneous injections.

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RESEARCH PAPERS

THE IDENTIFICATION OF THE CLINICALLY-IMPORTANT SULPHONAMIDES

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SINCE the discovery in 1935 of the chemotherapeutic value of the red azo-dye prontosil rubrum, and the subsequent proof that its activity *in vivo* is due to its conversion into sulphanilamide, an immense number of sulphonamide derivatives has been synthesised. The vast majority of these derivatives has found no place in medicine, and in this country some dozen only are in current clinical use.

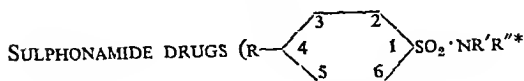
Owing to their relatively low toxicities, the sulphonamide drugs are unlikely suicidal agents, but during their therapeutic use toxic symptoms of varying severity not infrequently develop, necessitating clinical and chemical supervision. Excretion of the drugs occurs essentially *via* the urine, in which they are found either uncharged, in acetylated form, or to a lesser degree as the sulphates or glucuronates of derived phenols, the relative proportions of each form varying with the nature of the original substituent radical (Williams¹). Thus urine, or blood, is the usual material examined in the biochemical control of sulphonamide therapy, but the toxicologist may be further concerned with the isolation and identification of these drugs when occurring in viscera and medicinal preparations. From an analytical standpoint, a serious difficulty arises from the fact that no reactions specific for the typical sulphonamide linkage have been described. Quantitative determinations of the sulphonamide content of urine and blood are usually based upon such reactions of the free primary amino group as diazotisation (Fuller² Bratton, Marshall, Babbitt and Hendrickson³), production of the yellow Schiff's bases with *p*-dimethylaminobenzaldehyde (Werner⁴), or the indophenol reaction (Lapière⁵⁻¹²). Whilst such reactions have some merit of simplicity, it is evident that interference would result from the presence of other aryl primary amino compounds (Pons and Abel¹³), and clearly the method is ineffective with sulphonamides in which the primary amino group is substituted.

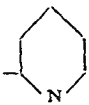
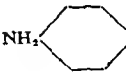

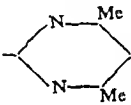

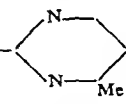
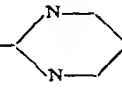




Methods for the qualitative identification of individual sulphonamides figure widely in the literature, and range from the synoptic schemes of Burkham¹⁴, Deniges¹⁵, Pesez¹⁶, and Hoffmann and Wilkens^{17,18}, to the colour reactions of Chavez¹⁹, Sample²⁰, and other workers and the crystal reactions suggested by Beck²¹, Dodson and Todd²², and Lapière⁵⁻¹². The present authors have repeatedly investigated the various methods hitherto described, but nevertheless have felt the need for a simple technique for identification of the drugs in the micro-quantities in which they may be encountered in toxicological analyses.

The number of sulphonamides is so large, whilst those of clinical importance are so relatively few, that any attempt at systematic identifi-

IDENTIFICATION OF SULPHONAMIDES

TABLE I



	R	R'	R''	M _p t (°C)
Sulphanilamide B P. Proseptasine Sulphacetamide B P.	$\text{NH}_2\text{--}$ $\text{C}_6\text{H}_4\text{CH}_2\text{NH--}$ $\text{NH}_2\text{--}$	--H --H --H	--H --H --COCH_3	165 175 183
Sulphaguanidine B P.	$\text{NH}_2\text{--}$	--H	--C-- NH NH_2	191
Sulphapyridine B P C	$\text{NH}_2\text{--}$	--H		191
Uleron . . .	$\text{NH}_2\text{--}$  $\text{SO}_2\text{NH--}$	--CH_3	--CH_3	194
Succinylsulphathiazole B P.	$\text{CH}_2\text{CO NH--}$ CH_2COOH	--H		195 (185)
Sulphadimidine	$\text{NH}_2\text{--}$	--H		199 (176)
Sulphathiazole B.P.	$\text{NH}_2\text{--}$	--H		201
Sulphamerazine	$\text{NH}_2\text{--}$	--H		236
Sulphadiazine B P	$\text{NH}_2\text{--}$	--H		255 d
Phthalylsulphathiazole	 CO NH-- COOH	--H		260 d
Soluseptasine	$\text{PhCHCH}_2\text{CHNH--}$ SO_2Na SO_2Na	--H	--H	—
Solupyrindine	$\text{PhCHCH}_2\text{CHNH--}$ SO_2Na SO_2Na	--H		—
Soluthiazole	$\text{PhCHCH}_2\text{CHNH--}$ SO_2Na SO_2Na	--H		—

* The drugs are listed under the name given in the British Pharmacopœia or British Pharmaceutical Codex, or if non-official, under a common trade name. The obsolescent azo-compounds prontosil rubrum and prontosil soluble are not included in this survey.

cation in the field as a whole would be unnecessarily cumbersome and would have little practical value. The investigations described in this publication, therefore, have been confined to those sulphonamide drugs commercially available at the present time in this country (Table I).

Residues isolated from medicinal preparations, or from viscera or other biological sources, are examined in a series of separate stages: (1) purification of crude residues, (2) provisional identification as a sulphonamide compound, (3) demonstration of the presence or absence of a free primary amino group, (4) simple crystal tests, (5) colour test indicative of the substituted sulphapyrimidines, (6) final conclusive identification by micromixed melting-point determination. The complete scheme provides a simple and rapid method for the identification of the listed sulphonamides, but in many instances characterisation may be achieved without inclusion of all the stages. It is emphasised that the object of the preliminary stages is essentially the indication of the likely compound, and whilst by the use of control experiments it is frequently possible to obtain a clear identification of an unknown sulphonamide by means of the preliminary tests alone, the ultimate proof should be by micromixed melting-point determination.

EXPERIMENTAL

Isolation of the sulphonamides. Sulphonamide therapy is of so recent introduction that few of the standard toxicological works have any mention of this group of drugs. Bamford²³, in a brief treatment of the sulphonamides, deals largely with methods of determination, and observes "Identification must depend on their isolation—often a difficult process—and examination of physical properties. Generally, however, the history of the case, combined with the results of the non-specific diazotisation and condensation reactions suffice to establish (or, more usually to confirm) the nature of the poison." The difficulties of isolation which this author so rightly mentions are largely attributable to the low solubilities of the sulphonamides in the water-immiscible organic solvents. Literature figures for the solubilities are by no means consistent, but the comparative record of available data (Table II) is useful, particularly when dealing with mixtures of sulphonamides.

In the Stas-Otto process, and also in the tungstic acid method described by Valov²⁴, sulphonamides are found in the ether extract of the aqueous acidic solution. The solubilities in ether, however, are so low that a small percentage only of the sulphonamide is recovered in this way, but in dealing with mixtures of sulphonamides with other compounds this is a definite advantage, as it frequently affords a ready means of separation. Complete removal from the aqueous acid medium may be effected by addition of half the volume of acetone, followed by thorough extraction with ether. For quantitative determination of total sulphonamides in material from biological sources, hydrolysis of the various derivatives is necessary. Sulphonamides generally, are present partly in the form of N_4 -acetyl derivatives, whilst the three disodium cinnamylidene bisul-

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phite compounds listed occur (Wien and Hampton²⁵) as the parent sulphonamides, their acetyl derivatives, and possibly also as the unchanged bisulphite compounds. Hydrolysis is readily accomplished by refluxing with 2N hydrochloric acid for 20 to 30 minutes.

Purification of crude residues. The low solubilities of the drugs are an undoubted advantage in connection with their purification. As a general method, heating with activated charcoal in acetone solution is found satisfactory; losses due to absorption are negligible, and a clean product, suitable for a preliminary melting point determination, is usually obtained.

Wide differences are found in the reported melting points of several sulphonamides. In the case of sulphadimidine, this variation is attributed

TABLE II
SOLUBILITIES OF SULPHONAMIDE DRUGS.*

	Water	2N sulphuric acid	2N sodium hydroxide	Alcohol	Ether	Chloroform	Acetone	Light Petroleum b pt 60° to 80° C
Sulphanilamide	1-125	Soluble	Soluble	1-37	1-600	1-4000	1-5	Insoluble
Sulphaguanidine	1-1000	Soluble	Insoluble	1-200	Insoluble	Insoluble	1-300	Insoluble
Sulphapyridine	1-3500	Soluble	Soluble	1-340	1-2200	1-1500	1-65	Insoluble
Sulphadiazine	1-13 000	Soluble	Soluble	1-1100	Insoluble	Insoluble	1-170	Insoluble
Sulphamerazine	1-6250	Soluble	Soluble	1-400	Insoluble	1-2000	1-60	Insoluble
Sulphadimidine	1-5000	Soluble	Soluble	1-200	1-5000	1-600	1-20	Insoluble
Sulphacetamide	1-150	Soluble	Soluble	1-15	1-600	1-1200	1-7	Insoluble
Sulphathiazole	1-2000	Soluble	Soluble	1-200	1-2400	1-375	1-23	Insoluble
Succinylsulphathiazole	1-4800	Slightly soluble	Soluble	1-100	Insoluble	Insoluble	1-110	Insoluble
Phthalylsulphathiazole	1-7500	Slightly soluble	Soluble	1-500	Insoluble	Insoluble	1-250	Insoluble
Proseptasine	1-32,000	Slightly soluble	Slightly soluble	1-145	1-320	1-1500	1-10	Insoluble
Uleron	1-50,000	Slightly soluble	Soluble	1-250	Insoluble	1-1000	1-20	Insoluble

* Solubilities in water are given numerically throughout. Organic solvent solubilities lower than 1 in 5000 are indicated as Insoluble.

(Northey²⁶) to the occurrence of unstable hydrates. A similar explanation may hold for other sulphonamides, and the figures in parenthesis in Table I may represent the melting points of these hydrates. It is further likely that this may also be the explanation of the wide differences in solubilities recorded in the literature.

Provisional identification as a sulphonamide. Using macro-quantities, identification by standard analytical methods presents no particular problem, but on a micro-scale the lack of a specific reaction for the sulphonamide grouping is a serious disadvantage. Lapière²⁷ has investigated the cobalt reaction described by Parri²⁸ in connection with the identification of barbiturates, and has found that certain sulphonamides, notably sulphathiazole, sulphapyridine and sulphadiazine, also give the characteristic purple-violet colour. Unfortunately, the reaction cannot be regarded as a general test for the presence of sulphonamides.

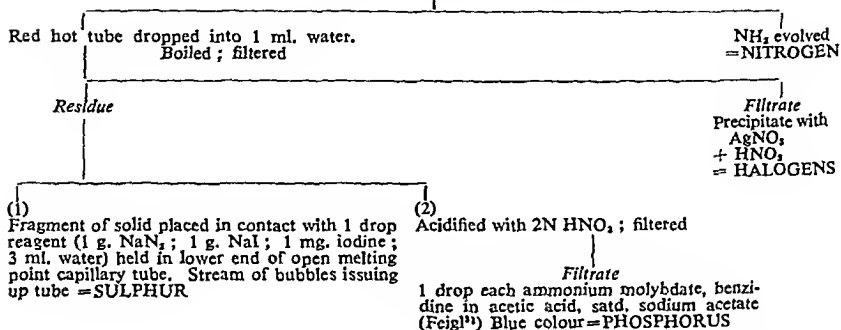
Provisional characterisation of isolated and purified residues as

sulphonamide compounds has been accomplished by the present authors in the following manner:

(i) Detection of elements by an adaptation of the micro-method described by Bennett, Gould, Swift and Niemann²⁹. The procedure is given in detail in Table III.

TABLE III
MICRO-IDENTIFICATION OF ELEMENTS

1 mg. residue + 5 times weight
of Zn dust - CaO mixture (1:1)
heated in 4.5 cm. x 0.5 cm. tube



(ii) If nitrogen and sulphur only are detected sulphonamides may be present, and the effect of heating in admixture with soda-lime is then investigated according to the scheme in Table IV.

TABLE IV
DECOMPOSITION ON HEATING WITH SODA-LIME

0.1 mg. residue heated with equal
quantity soda-lime



The clinically-important sulphonamides listed are all primary amino derivatives, and on fusion with soda-lime are decomposed with the production of ammonia or readily volatile amines, aniline bases and sodium sulphite; sulphathiazoles and the cinnamylidene bisulphite compounds yield, in addition, sodium sulphide. Thus, by the method in Table IV, positive tests for ammonia, aniline and sulphite provide presumptive evidence of the presence of a sulphonamide; sulphide in addition suggests the sulphathiazoles or cinnamylidene bisulphite compounds.

Individual sulphonamides differ considerably in the rate at which the various products are formed, but by using a standard technique the reactions with quantities of the order of 0.1 mg. are equally as satisfactory as those utilising larger amounts of material.

0.1 mg. of the test material intimately mixed with an equal weight

IDENTIFICATION OF SULPHONAMIDES

of soda-lime is gently warmed in a small test tube (4.5 cm. x 0.5 cm.). The issuing vapours are tested with a pointed strip of moist red litmus paper introduced just inside the tube. Stronger heat is now applied until an oily distillate accompanied by white fumes approaches to within approximately 0.5 cm. from the open end of the tube. The tube is placed on a white tile, and a pointed strip of filter paper moistened with a 2 per cent. solution of furfural in glacial acetic acid carefully introduced. In the presence of aniline (which may also frequently be detected by odour) a distinct reddish-pink band appears around the tube in the region of the oily distillate. At this stage one drop of saturated aqueous mercuric chloride solution is added to the solid residue in the tube, in order to obviate interference from hydrogen sulphide on subsequent acidification; a fragment of the treated residue is tested for sulphide by the iodine-azide reaction. The contents of the tube are finally heated with dilute hydrochloric acid, and the issuing vapours tested with a pointed strip of moistened filter paper impregnated with starch and potassium iodate, when a blue colour develops in the presence of sulphur dioxide. The results of this test with the listed sulphonamide drugs are summarised in Table V. It should be emphasised that this procedure does

TABLE V
REACTIONS OF SULPHONAMIDES

	Decomposition by soda-lime fusion				Furfural reaction for free NH_2
	Ammonia	Aniline	Sulphite	Sulphide	
Sulphanilamide	—	+	+	—	+
Prosectasine	—	—	—	—	—
Sulphacetamide	—	—	+	—	+
Sulphaguanidine	—	+	—	—	+
Sulphapyridine	+	+	—	—	+
Uleron	—	—	—	—	+
Succinylsulphathiazole	—	—	—	+	+
Sulphadimidine	—	—	—	—	+
Sulphathiazole	+	—	—	+	+
Sulphamerazine	+	—	—	—	+
Sulphadiazine	+	—	+	—	+
Phthalylsulphathiazole	—	—	—	+	—
Soluseptazone	—	—	—	+	—
Solupyrindine	—	—	—	+	—
Soluthiazole	—	—	—	+	—

not provide unequivocal proof that the material is a sulphonamide, but positive reactions here coupled with indications of unusual insolubility is strong presumptive evidence.

Detection of free NH_2 groups. Aryl primary amino groups may be detected by a variety of reactions, of which diazotisation followed by coupling is perhaps the most usual. The information can however be obtained equally satisfactorily, and much more rapidly, by means of the furfural condensation. 0.1 mg. to 1 mg. of material is placed in a white porcelain dish and treated with 1 drop of 2 per cent. solution of furfural in glacial acetic acid. The liquid is allowed to evaporate spontaneously, during which process a free primary amino group is indicated by the production of an intense red colour, rapidly turning reddish-violet.

The reactions of the sulphonamides are listed for convenience in Table V. It is noteworthy that in the case of the pyrimidine derivatives, the colour is appreciably slower in developing, and may not be apparent until evaporation is complete. Of the listed sulphonamides, positive tests for both sulphide and free -NH_2 are given by one compound only, sulphathiazole. Similarly, one compound only, proseptasine, gives negative tests for both sulphide and free -NH_2 .

Crystal tests. Since the sulphonamides are, in general, soluble both in acid and alkali, acidification of an ammoniacal solution as recommended for the identification of barbiturates (Turfitt³⁰) is valueless. It has been found, however, that by a variation in the technique, crystals of the sulphonamides may in most cases be readily obtained. The

TABLE VI
CRYSTAL FORMATION

	Strong solution of ammonia 0.880	Acetic acid vapour
Sulphanilamide ...	Soluble with difficulty	* No peripheral precipitation. Long fine needles radiate from undissolved particles; also occasional hexagonal forms
Proseptasine ...	Insoluble	—
Sulphacetamide ...	Very readily soluble	—
Sulphaguanidine ...	Insoluble. Slightly soluble hot; long needles on cooling.	—
Sulphapyridine ...	Readily soluble	* Rapid peripheral precipitation. Characteristic 'banded' crystals, with some hexagonal and arborescent needle formations.
Uleron ..	Readily soluble	* Rapid peripheral precipitation. Minute globules with characteristic 'dumb-bell' shaped crystals. Wrinkled surface skin develops over drop.
Succinylsulphathiazole	Very readily soluble	—
Sulphadimidine ...	Readily soluble	Rapid peripheral precipitation. Minute globules giving large rosettes of brownish needles. Wrinkled surface skin develops over drop.
Sulphathiazole ...	Very readily soluble	* Slow peripheral precipitation. Minute drops coalescing rapidly into large globules, and giving arborescent needle-shaped growths.
Sulphamerazine ...	Readily soluble. On evaporation 'curved' crystals usually obtained	Rapid peripheral precipitation. Three crystalline forms usually obtained: (a) rods, often with bifurcated ends, followed by (b) 'twinned' crystals, and finally (c) aggregates of 'curved' crystals. Any one of these forms is characteristic of the compound.
Sulphadiazine ...	Readily soluble	Fairly rapid peripheral precipitation. Needles individually or in clusters.
Phthalylsulphathiazole	Readily soluble	* Slow peripheral precipitation. Thin massed needle rosettes. Wrinkled surface skin forms over drop.
Soluseptasine...	Very readily soluble	—
Solupyridine ...	Very readily soluble	—
Soluthiazole ...	Very readily soluble	—

* The same crystalline forms are obtained on spontaneous evaporation of the cold ammoniacal solution.

IDENTIFICATION OF SULPHONAMIDES

crystalline forms are generally highly distinctive, and it is frequently possible to identify a sulphonamide by means of this test alone.

0.1 mg. of material is finely powdered on a microscope slide and 1 drop of 0.880 ammonia added. The mixture is stirred thoroughly with a fine glass rod and observation made of the ease or difficulty of solution. A drop of glacial acetic acid on the end of a glass rod is then held just above the surface of liquid until a white turbidity appears at the margin of the drop, or, as in the case of sulphaniilamide, crystals appear within the drop itself, when the slide is examined microscopically at a magnification of approximately X 50.

A general description of the crystals obtained with the various sulphonamides is given in Table VI, whilst for reference purposes the characteristic forms are illustrated in Figures 1 to 11.

It is regarded as essential that control crystal tests should be made with authentic material, and it is further recommended that after examination of the crystals these should be redissolved by treatment with ammonia vapour or solution, reprecipitated with glacial acetic vapour, and again examined. No loss of material is incurred during this repeated test, but confirmation of the typical crystalline form is obtained.

Vanillin reaction for substituted sulphapyrimidines. When warmed with vanillin and concentrated sulphuric acid the majority of the sulphonamides give a yellowish-green colour; the methylpyrimidine compounds sulphamerazine and sulphadimidine however, give an intense bright red colour. This property has been found useful as a confirmatory test, and has been adapted for micro-quantities.

A quantity of vanillin of the order of 0.01 mg. is mixed on a microscope slide with 1 small drop of concentrated sulphuric acid. Into the liquid is dropped approximately 0.01 mg. of the sulphonamide, and the mixture warmed over a micro-flame until fumes are just observable. The slide is placed upon a white tile, and the presence or absence of a red colour arising from the sulphonamide particles is noted.

Final mixed melting-point. The information derived from the foregoing tests is invariably conclusive for any one of the listed sulphonamides, but the conclusion should be checked by a mixed melting-point with a specimen of the indicated compound.

MIXTURES OF SULPHONAMIDES

With the introduction of proprietary mixtures, e.g. sulphatriad, containing sulphathiazole, sulphadiazine and sulphamerazine, the toxicologist may be required to identify the individual components of such mixtures, either in biological material or during the analysis of actual tablets.

It is clearly essential in such cases to effect a preliminary separation of the components. This may be achieved satisfactorily on the basis of solubility differences (Table II), subsequent identification of each fraction being accomplished by the described methods.

The authors have encountered no insuperable difficulties in the qualitative analysis of mixtures treated in this manner.

SUMMARY

1. A scheme is described for the identification of all the sulphonamide drugs at present available in this country.
2. The six stages of the process are simple and rapid operations, involving no unusual reagents or apparatus:
 - (i) purification of the crude material by charcoal treatment in acetone solution, followed by melting-point determination.
 - (ii) preliminary identification as a sulphonamide compound by decomposition with soda-lime.
 - (iii) detection of free primary amino group by condensation with furfural.
 - (iv) simple crystal tests based on precipitation from ammonia solution by acetic acid vapour.
 - (v) vanillin confirmatory test for sulphamerazine and sulphadimidine.
 - (vi) mixed melting-point determination.

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IDENTIFICATION OF SULPHONAMIDES



FIG. 1. Sulphanilamide.

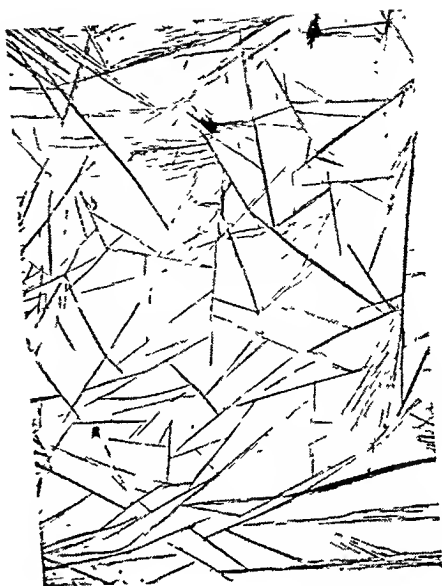


FIG. 2. Sulphaguanidine.



FIG. 3. Sulphapyridine.

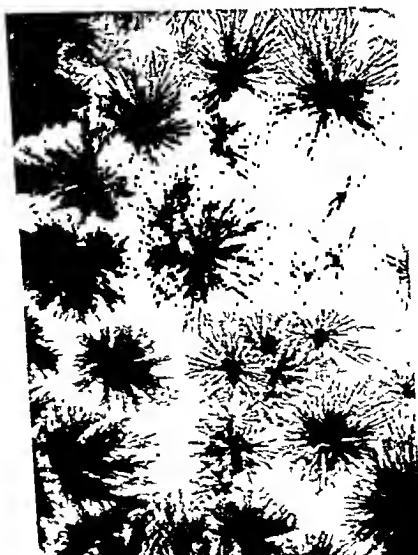


FIG. 4. Sulphadimidine.

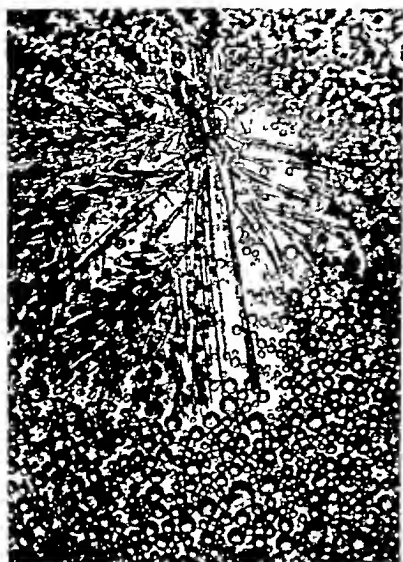


FIG. 5. Sulphathiazole.



FIG. 6. Sulphamerazine (1).



FIG. 7. Sulphamerazine (2).



FIG. 8. Sulphamerazine (3).

IDENTIFICATION OF SULPHONAMIDES



FIG. 9. Sulphadiazine.

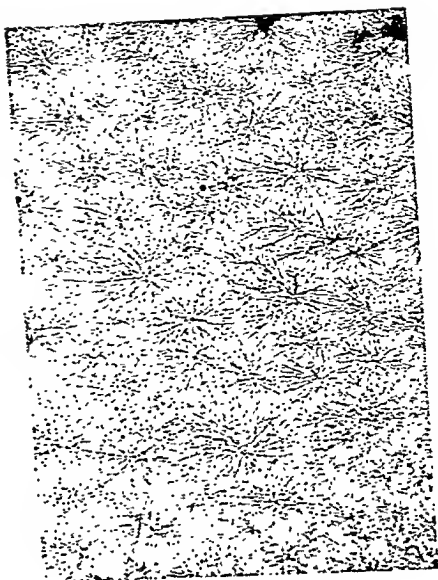


FIG. 10. Phthalysulphathiazole.

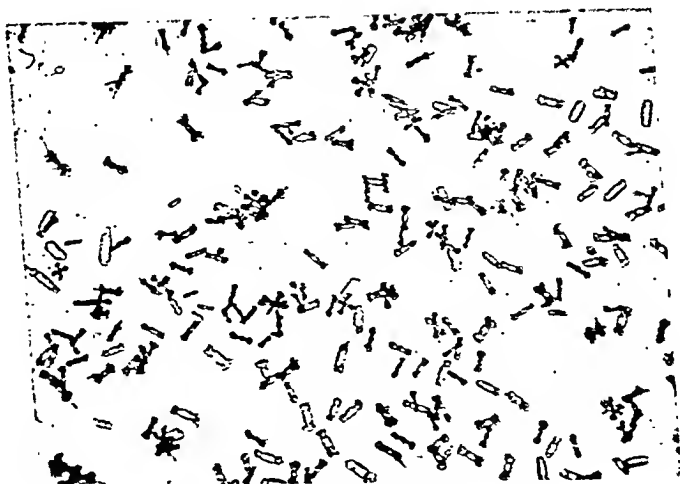


FIG. 11. Uleron.

The magnification of Figures 1-10 is approximately $\times 40$, and of Figure 11, $\times 50$.

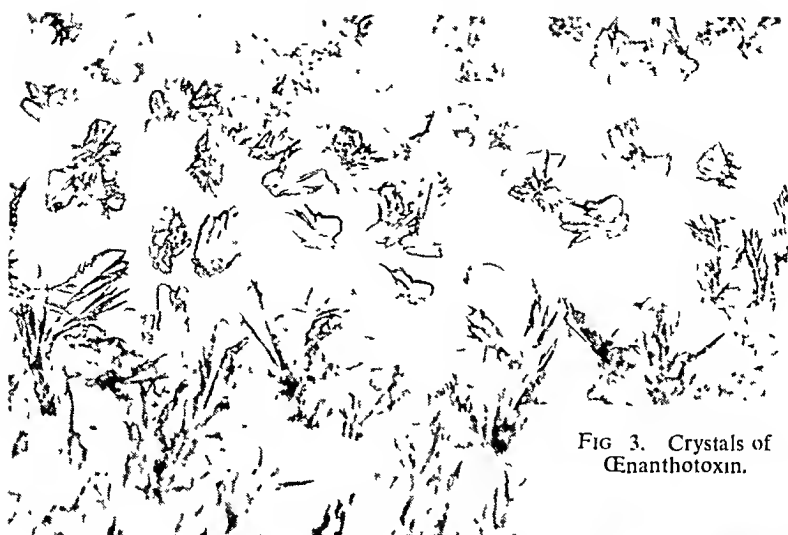


FIG 3. Crystals of Ceanothotoxin.



FIG 4 Ceanothotoxin after recrystallisation from ethyl alcohol



FIG 1 Chromatogram of toxic principle of *Ceanotho cuneata*

THE ISOLATION OF THE TOXIC PRINCIPLE OF *ÆNANTHE CROCATÀ*

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INTRODUCTION

ÆNANTHE CROCATÀ Hemlock Water Dropwort, belonging to the family Umbelliferae, is common in wet places all over Western Europe, including the British Isles. Its toxicity has been known for a great many years, Linnaeus having noted it in Sweden in 1746 (Skarman¹), while Orfila² quotes a number of instances of poisoning in the 17th and 18th centuries. Witthaus³ cites 159 cases, 42 of them (i.e., 26 per cent.) being

fatal, while there are numerous references to the subject in more recent medical literature (Thomas⁴; McGarth⁵). Holmes⁶ refers to *Ænanthe crocata* as the most poisonous plant in England, while Fenton and Robertson⁷ state that it is responsible for more stock poisoning than any other. The frequency with which cases of poisoning occur is probably because it has a pleasant taste, and an attractive smell, rather like celery.

In spite of its well-established toxicity, very little work has been done on the chemistry of the plant. Cormerais and Pihan-Dufeillay⁸ found the active ingredient to be contained in a resinoid material in the root, while Gerding⁹ discovered a similar material in *Æ. fistulosa*. By purifying an ethyl alcoholic extract of the root by dissolving in ether, washing with sodium hydroxide and precipitating with light petroleum, Pohl¹⁰ obtained a neutral resinous substance, which he named ænanthotoxin, and to which he assigned the formula $C_{17}H_{22}O_3$. Tutin¹¹ isolated several non-toxic substances (triacontane, hentriacontane and ipuranol) from an ethyl alcoholic extract of the root, but he also found that the toxicity was associated with the ether-soluble neutral resin. He considered that "Pohl's ænanthotoxin" was not a pure substance. We are not aware of any other chemical investigation of value.

EXPERIMENTAL

Extraction. As a preliminary experiment, ethyl alcoholic extracts of roots, seeds and green

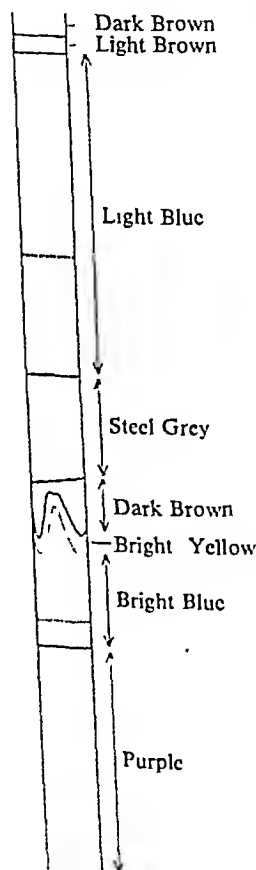


FIG 2. Diagram showing sections of Chromatogram

stems were made, and tested for toxicity by intra-peritoneal injection in mice. Only the extract from the root proved lethal, so investigation was confined to this part of the plant. The roots were obtained from the banks of the Thames near Kew, and passed, while still fresh, through an ordinary kitchen mincer. An extract made with 0.9 per cent. sodium chloride solution showed no activity, while there was little to choose in toxicity between extracts made with ethyl alcohol, chloroform or ether. The latter solvent was chosen owing to the ease of removal. Air-dried roots extracted with either ethyl alcohol or ether gave less toxic preparations.

6 kg. of freshly minced roots were gently refluxed in a 20-l. bolthead flask for a total time of 12 hours with 11 l. of ether. The residue was washed with 6 l. of ether, the washings added to the extract, and the whole dried over anhydrous sodium sulphate. The ether was then distilled off, leaving 25 g. of a toxic brown oily residue, with an A.L.D. in mice of approx. 25 mg./kg. This oil was dissolved in 30 ml. of ether and 600 ml. of light petroleum was added. This threw down 17.5 g. of a toxic resinous material. The fraction remaining in the light petroleum was not toxic. The resinous precipitate was dissolved in 300 ml. of ether and shaken with 300 ml. of 40 per cent. sodium hydroxide, then with 300 ml. of 8 per cent. sodium hydroxide solution, and finally washed with saturated sodium sulphate solution (it having been found that water gave troublesome emulsions) until the washings were neutral. The sodium hydroxide extract, neutralised with hydrochloric acid, threw down a dark resinous material that was not toxic. The ethereal solution was dried over anhydrous sodium sulphate and evaporated to dryness, yielding 8 g. of light brown viscous oil, which was highly toxic. This oil deposited a few crystals on standing, but insufficient for investigation.

CHROMATOGRAPHY

Chromatography in daylight was unsatisfactory, but on elution on alumina columns under ultra-violet light a series of coloured bands was given by most organic solvents. The best separation was obtained with a mixture of dry benzene and ethyl alcohol (99:1). Therefore a solution of the light brown oil in benzene/ethyl alcohol (99:1) was placed on an alumina column and developed with the same mixed solvent. The bands seen under ultra-violet light were removed, eluted separately with ethyl alcohol and the extracts tested on mice. The toxicity was found to be associated with a band which gave a steel-grey fluorescence (Figs. 1 and 2).

The 5 g. of pale yellow oily material obtained on evaporating the extract from this band deposited oily crystals on standing. These were recrystallised by dissolving in the minimum of chloroform and cooling to -15°C . 400 mg. of colourless crystals was obtained, which were highly toxic (*v. infra*). They can be purified, either by recrystallisation from chloroform, methyl alcohol or benzene, dissolving at room temperature.

TOXIC PRINCIPLE OF *ÆNANTHE CROCAT*

and cooling as above, or by chromatography on an alumina column, eluting with benzene/ethyl alcohol (99:1), when a single steel-grey zone is obtained. These purifications do not alter the melting point or crystalline habit.

PROPERTIES

Crystalline "ænanthotoxin" prepared by this method forms small colourless irregular crystals, (Fig. 3) m.pt. 80° to 81°C., insoluble in water, light petroleum, alkalis and dilute mineral acids, but freely soluble in ether, ethyl alcohol and chloroform. If dissolved in ethyl alcohol, and the solvent allowed to evaporate, the substance crystallises in flat plates, many of them with a characteristic "bullet-shaped" appearance (Fig. 4). Found C, 71.75; H, 7.29; O, 20.96 per cent. Molt. wt. (cryoscopic in benzene) 292 $C_{16}H_{22}O_4$ requires C, 71.50; H, 7.43; O, 21.16, Mol. wt. 302.

Nitrogen is absent. The substance is extremely unstable, changing comparatively rapidly into a brown insoluble resinous material, decomposing without melting above 200°C., and possessing no pharmacological activity. This change is accelerated by a high temperature and by oxygen. At 4°C. under oxygen-free nitrogen ænanthotoxin is much more stable, only a slight yellow colour developing in a period of weeks. The specific rotation in chloroform is + 14.7°. Ænanthotoxin gives an immediate black colour with concentrated sulphuric acid.

PHARMACOLOGY

Ænanthotoxin, in the form of an emulsion, was injected intraperitoneally into white mice. In the earlier preparations the emulsion had been prepared by dissolving the substance in 0.5 ml. of ethyl alcohol

TABLE I

Results of injecting 0.5 ml. of lecithin-saline suspension of crystalline ænanthotoxin containing the dose shown, intraperitoneally into white mice, c.18 g. weight. C—Interval before onset of convulsions. (In minutes from time of injection). D—Time elapsing before death. S—Survived.

Mouse No.		1	2	3	4	5	Average
Dose							
25 mg.	{C	3	3	3	2	3	2.8
	{D	22	10	27	21	7	17.4
12 mg.	{C	3	4	4	3	4	3.4
	{D	23	51	28	51	7	32.0
0.6 mg.	{C	13	8	4	4	5	6.8
	{D	26	45	44	42	59	43.2
0.3 mg.	{C	11	19	13	20	16	15.8
	{D	83	58	57	88	27	62.6
0.15 mg.	{C	28	20	32	42	23	29.4
	{D	5	38	5	5	32	
0.07 mg.	{C	
	{D	
0	{C	
	{D	

Controls received 0.5 ml. lecithin saline only.

This gives an A.L.D. of approximately 0.83 mg./kg. Pohl's ænanthotoxin killed one rabbit in 105 minutes at a dose of 24 mg./kg.

and pouring this into 9.5 ml. of 0.9 per cent. sodium chloride. In the case of the crystalline product, the emulsion produced by this method was unstable, owing to the removal of some emulsifying agent by the purification. The emulsion of crystalline *œnanthotoxin* for injection was made by pouring an ethyl alcoholic solution into a 0.1 per cent. solution of lecithin in 0.9 per cent. sodium chloride solution.

The results of the experiment on mice with the crystalline substance are given in Table I.

SIGNS

Shortly after injection the animal becomes perceptibly less active, respiration is accelerated but the animal shows no sign of distress. 1 to 20 minutes after injection the animal becomes restless and adopts the characteristic feeding posture with excessive movement of the fore limbs.

Soon tremors of varying intensity are observed which may be confined to only local regions, but more frequently are general. The onset of convulsions may be sudden, but it is usually preceded by a generalised tremor of the whole body. Depending on the dose the convulsive movements vary from those involving tonic contractions and rolling, to wild jumping movements. The convulsive stage varies in its duration: usually several convulsions follow in rapid succession, but occasionally an interval of some minutes may intervene between any two.

The terminal phase is invariably heralded by pedalling movements of the hind limbs and irregular vigorous movements of the fore limbs. Abduction of the digits is marked, and always accompanies the pedalling movements. At this stage hæmorrhages from the buccal cavity may be observed accompanied by trismus of the jaw muscles. Prior to death the animal assumes a characteristic posture, lying on one side with the fore limbs acutely flexed and the hind limbs rigidly extended to the full. Death follows.

These symptoms correspond closely with those recorded in cases of poisoning in man and domestic animals following the ingestion of the roots of *œnanthe crocata*.

SUMMARY

1. A method is described for the preparation of highly toxic crystals from water dropwort root. Previous workers have only reported oils or resins of far less toxicity.

2. These can be purified by recrystallisation, or by chromatography on an alumina column, when a single steel-grey zone is obtained. These purifications do not alter the melting point or the crystalline habit.

3. The crystalline material, m.pt. 80° to 81° C., is insoluble in water, light petroleum, alkalis, and diluted mineral acids, but readily soluble in chloroform, ethyl alcohol and ether.

4. The crystals are extremely unstable, yielding an insoluble infusible resinous material, with no pharmacological activity.

TOXIC PRINCIPLE OF *ŒNANTHE CROCATÁ*

5. If kept under nitrogen at 4°C., the crystals can be preserved for weeks with little loss of activity.

6. Death after characteristic convulsions follows the intraperitoneal injection of an emulsion of the crystalline material into mice.

7. The A.L.D. is 0.83 mg./kg. of body weight.

We wish to express our thanks to Mr. G. Knight, of the Beaumont Hospital, for the loan of apparatus and to Mrs. King for her drawing of Figure 2. We are also indebted to Mr. Burgess for the photo-micrographs, and to our technician, Mr. Waterman, for his help and for the collection of material.

The photograph of the chromatographic column was taken at the Optical Department of the Medical Research Council, Hampstead, and to Dr. Smiles, of this unit, we wish to accord our thanks. Thanks are also due to Dr. Klyne, of the Postgraduate Medical School, for the use of the micropolarimeter.

Our thanks are also due to Professor E. C. Amoroso, Head of the Department of Physiology, for his sympathetic interest and helpful suggestions during the course of this investigation.

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and pouring this into 9.5 ml. of 0.9 per cent. sodium chloride. In the case of the crystalline product, the emulsion produced by this method was unstable, owing to the removal of some emulsifying agent by the purification. The emulsion of crystalline œnanthotoxin for injection was made by pouring an ethyl alcoholic solution into a 0.1 per cent. solution of lecithin in 0.9 per cent. sodium chloride solution.

The results of the experiment on mice with the crystalline substance are given in Table I.

SIGNS

Shortly after injection the animal becomes perceptibly less active, respiration is accelerated but the animal shows no sign of distress. 1 to 20 minutes after injection the animal becomes restless and adopts the characteristic feeding posture with excessive movement of the fore limbs.

Soon tremors of varying intensity are observed which may be confined to only local regions, but more frequently are general. The onset of convulsions may be sudden, but it is usually preceded by a generalised tremor of the whole body. Depending on the dose the convulsive movements vary from those involving tonic contractions and rolling, to wild jumping movements. The convulsive stage varies in its duration: usually several convulsions follow in rapid succession, but occasionally an interval of some minutes may intervene between any two.

The terminal phase is invariably heralded by pedalling movements of the hind limbs and irregular vigorous movements of the fore limbs. Abduction of the digits is marked, and always accompanies the pedalling movements. At this stage hæmorrhages from the buccal cavity may be observed accompanied by trismus of the jaw muscles. Prior to death the animal assumes a characteristic posture, lying on one side with the fore limbs acutely flexed and the hind limbs rigidly extended to the full. Death follows.

These symptoms correspond closely with those recorded in cases of poisoning in man and domestic animals following the ingestion of the roots of *œnanthe crocata*.

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THE ABSORPTION SPECTRA AND IONIC DISSOCIATION OF THIOURACIL DERIVATIVES WITH REFERENCE TO THEIR ANTI-THYROID ACTIVITY

By R. E. STUCKEY

From The British Drug Houses, Limited, London

Received March 23, 1949

A NUMBER of derivatives of thiourea and especially the substituted thiouracils inhibit thyroid activity in animals^{1,2}; in particular thiouracil and methylthiouracil have been used in the treatment of hyperthyroidism in man. Miller, Roblin and Astwood³ studied the reaction between iodine and 2-thiouracil and found that at pH 7.4 in the presence of sodium bicarbonate a disulphide from two molecules of thiouracil was formed. The absorption of iodine was such that tyrosine and casein were protected from iodination under these conditions in the presence of 2-thiouracil. This was held to support the hypothesis that thio-derivatives may prevent hormone synthesis in the thyroid gland by blocking the iodination of hormone precursors. Doubts, however, have been expressed by Rimington and Lawson⁴ concerning the validity of this reaction ($2 \text{ RSH} + \text{I}_2 \rightleftharpoons \text{RS.SR} + 2 \text{ HI}$) as a mechanism owing to the activity of sulphonamide derivatives which have no free -SH group.

Williams and Kay⁵ found that the activity of thiouracil was distinctly decreased or in some instances lost by the addition of methyl or ethyl substituents on the nitrogen atoms or by the addition of substituents on the sulphur atom. Since both of these alterations to the molecule are connected directly or indirectly with the ionisable hydrogen atom it seems probable that ionisation is an important factor in thiouracil activity. It was therefore decided to investigate the dissociation of 2-thiouracil and 2-thio-4-methyluracil and to try and determine the probable structure of the resulting ion. With the latter object in view the methylated thiol structures corresponding to 2-thio-4-methyluracil, namely, 2-methylmercapto-4-methyl-6-oxypyrimidine and 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine, were prepared and their ultra-violet absorption spectra determined under varying pH conditions. At the same time the dissociation of the compounds prepared was investigated by means of an electrometric titration.

EXPERIMENTAL

Absorption Spectra. The absorption curves were determined at varying pH values using a Beckman photoelectric spectrophotometer. Samples of 2-thiouracil and 2-thio-4-methyluracil of appropriate strengths were dissolved in potassium dihydrogen phosphate-sodium hydroxide buffer solutions and examined in a 1 cm. cell. Solutions of high and low pH were obtained by dissolving in carbon dioxide-free water and adding sodium hydroxide or hydrochloric acid until the required pH was obtained as indicated by pH meter; solutions obtained in this manner were used immediately.

Electrometric Titrations. These were carried out in general by running N/10 aqueous sodium hydroxide into solutions of thiouracil

ABSORPTION SPECTRA OF THIOURACIL DERIVATIVES

derivatives in 50 per cent. aqueous ethyl alcohol, the pH of the solution being determined at intervals by a standard Cambridge pH meter. A typical result is shown for 2-thio-4-methyluracil:

Per cent. neutralised	10	20	30	40	50	60	70	80
pK _a
	8.6	8.5	8.5	8.6	8.5	8.5	8.4	8.4

The temperature throughout the pH measurements was $18^{\circ} \pm 2^{\circ}\text{C}$. The results are not corrected for the effects of alcohol.

Preparation of the methylated derivatives.

2-Methylmercapto-4-methyl-6-oxypyrimidine was prepared by methylation of 2-thio-4-methyluracil in sodium ethylate using methyl iodide according to List⁶. After crystallisation from alcohol it had m.pt. 220°C . Di-methylation of 2-thio-4-methyluracil, by the method of Wheeler and MacFarland⁷ produced 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine which after crystallisation from alcohol had m.pt. 94°C . The constitution of the methylated compounds was proved by Wheeler and MacFarland (*loc. cit.*) since on heating with hydrochloric acid 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine gave 1:4-dimethyluracil.

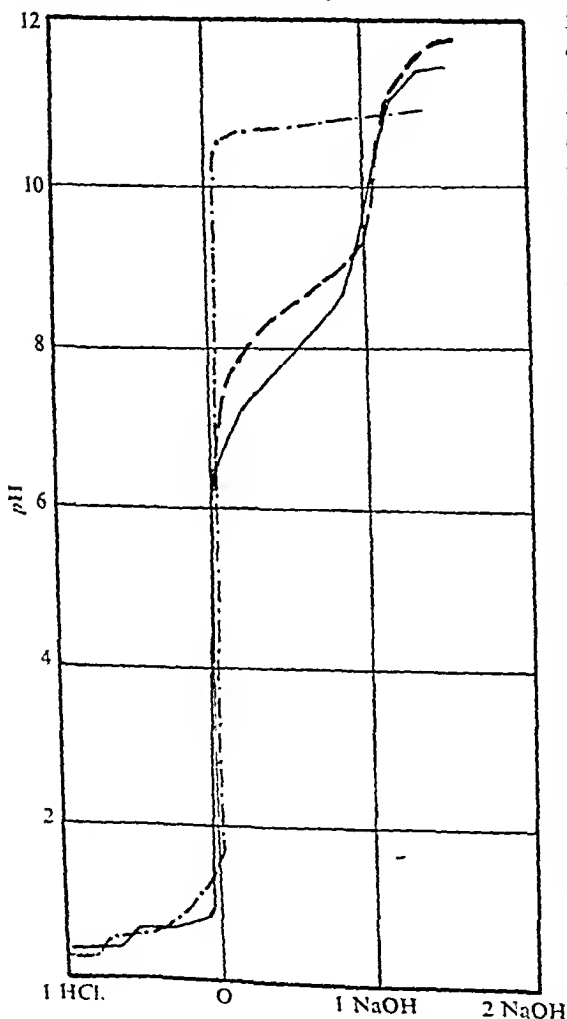


FIG. 1. Electrometric titration curves:

- 2-thio-4-methyluracil;
- 2-methylmercapto-4-methyl-6-oxypyrimidine;
- · - · 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine.

RESULTS

The results in general are of interest in showing the close correlation between dissociation as shown by the pH curves and changes in absorption

spectrum, i.e. structural changes are due primarily to ionisation. Thus 2-thio-4-methyluracil possesses a potentiometric titration curve (Fig. 1) in aqueous ethyl alcohol with a break corresponding to an acidic dissociation

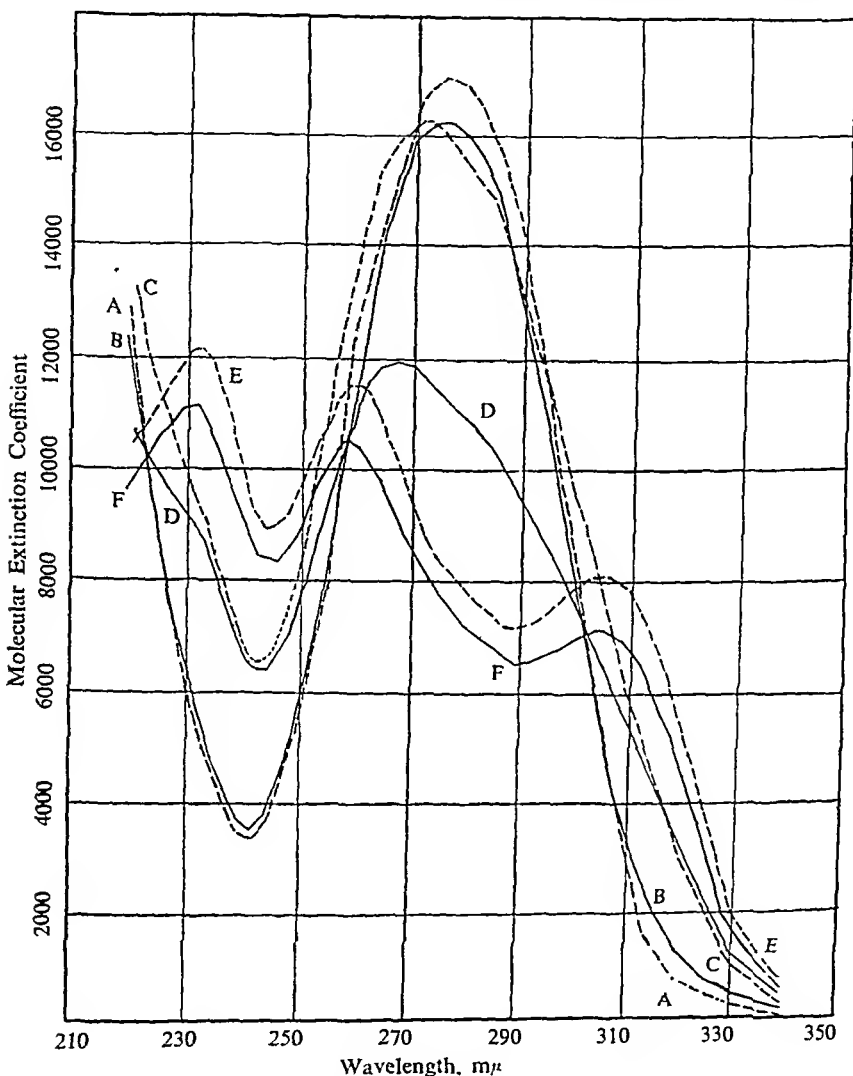


FIG. 2. Absorption spectra of 2-thio-4-methyluracil. A, at pH 2; B, at pH 7; C, at pH 8; D, at pH 9; E, at pH 10; F, at pH 12.

constant of pH 8.5. The spectra of this compound (Fig. 2) show minor differences only at pH values up to 7, and at pH 8 the main peak λ_{\max} ca. 270 mμ ϵ_{\max} ca. 16,000 is still not appreciably changed. At higher pH values, corresponding to increasing ionisation three peaks become evident, a transition curve occurring at pH 9.

ABSORPTION SPECTRA OF THIOURACIL DERIVATIVES

2-Methylmercapto-4-methyl-6-oxypyrimidine does not show any main peak ϵ_{\max} ca. 16,000 as for 2:thio-4-methyluracil, and the changes are not so fundamental (Fig. 3). A break in the pH curve (Fig. 1) between pH 0 and pH 2 corresponds to a change in absorption spectra at these

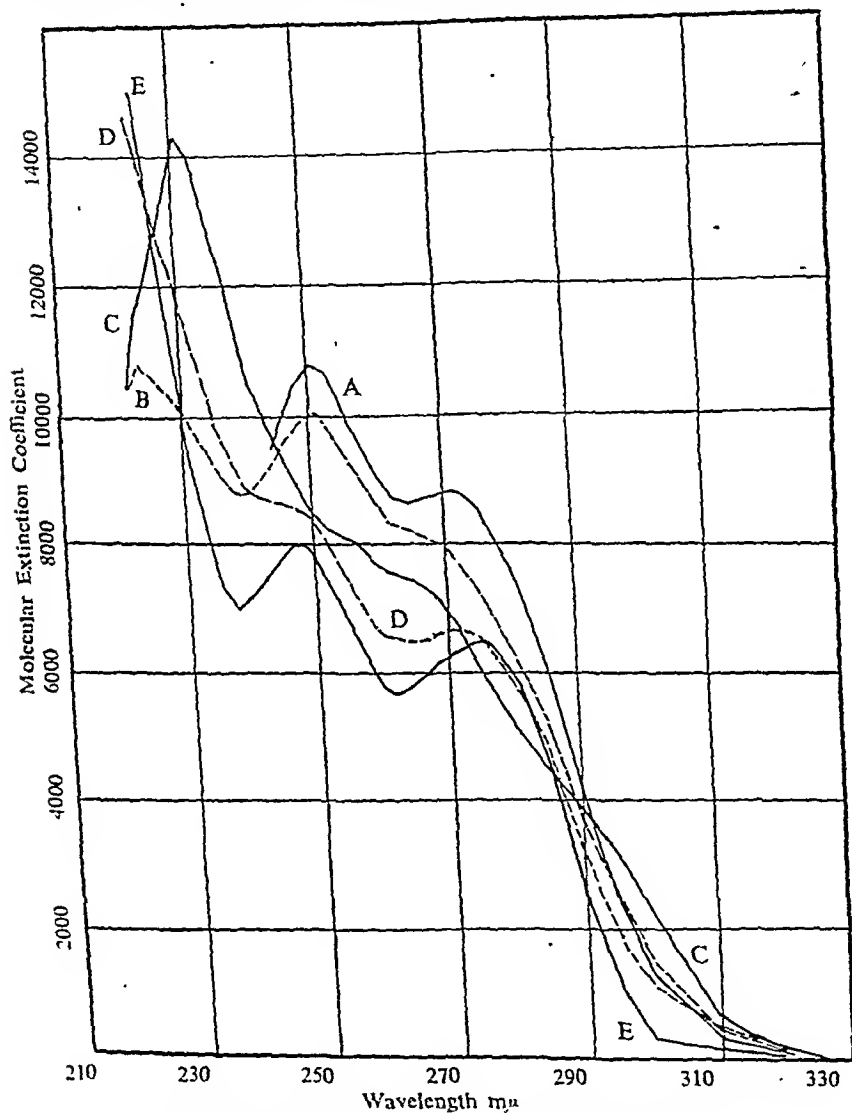


Fig. 3. Absorption spectra of 2-methylmercapto-4-methyl-6-oxypyrimidine. A, at pH 0; B, at pH 1; C, at pH 2; D, at pH 8; E, at pH 9.

values. The electrometric titration results for this compound show it to be a weak acid, having a pK_a value of 7.9.

1:4-Dimethyl-2-methylmercapto-6-oxypyrimidine shows absorption changes only at pH values less than 2 (Fig. 4), again corresponding to a

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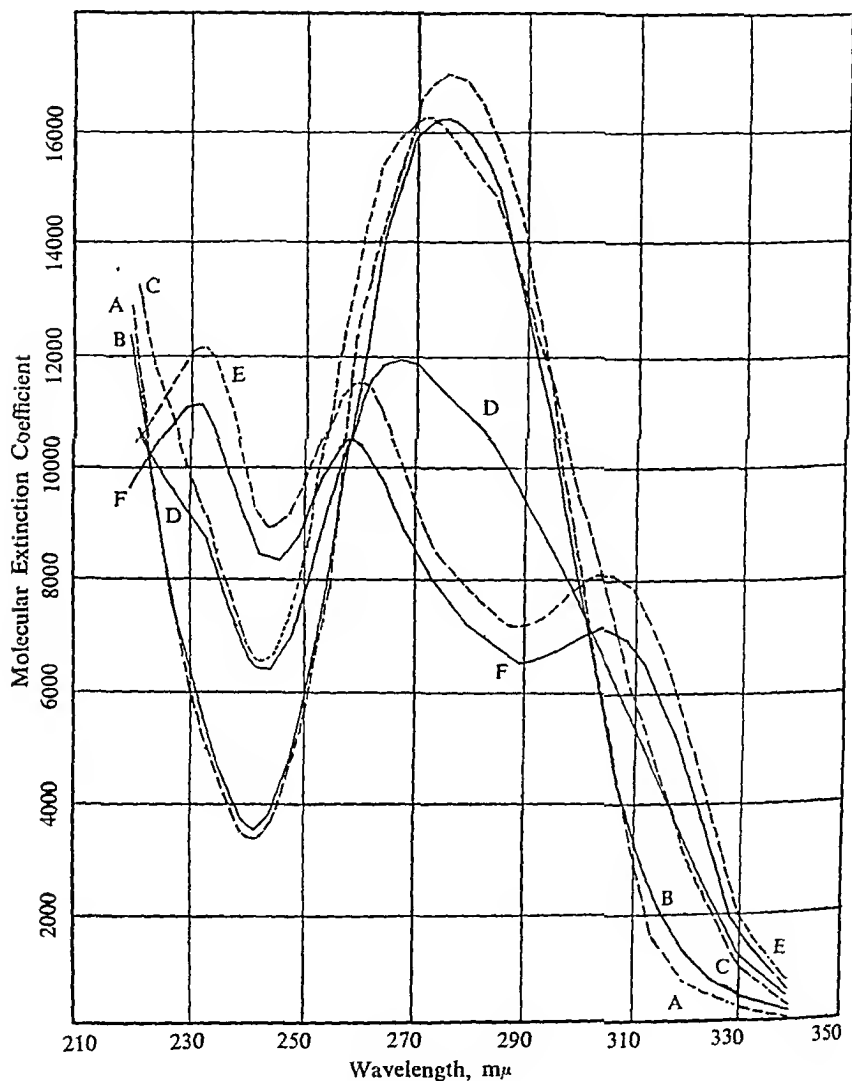


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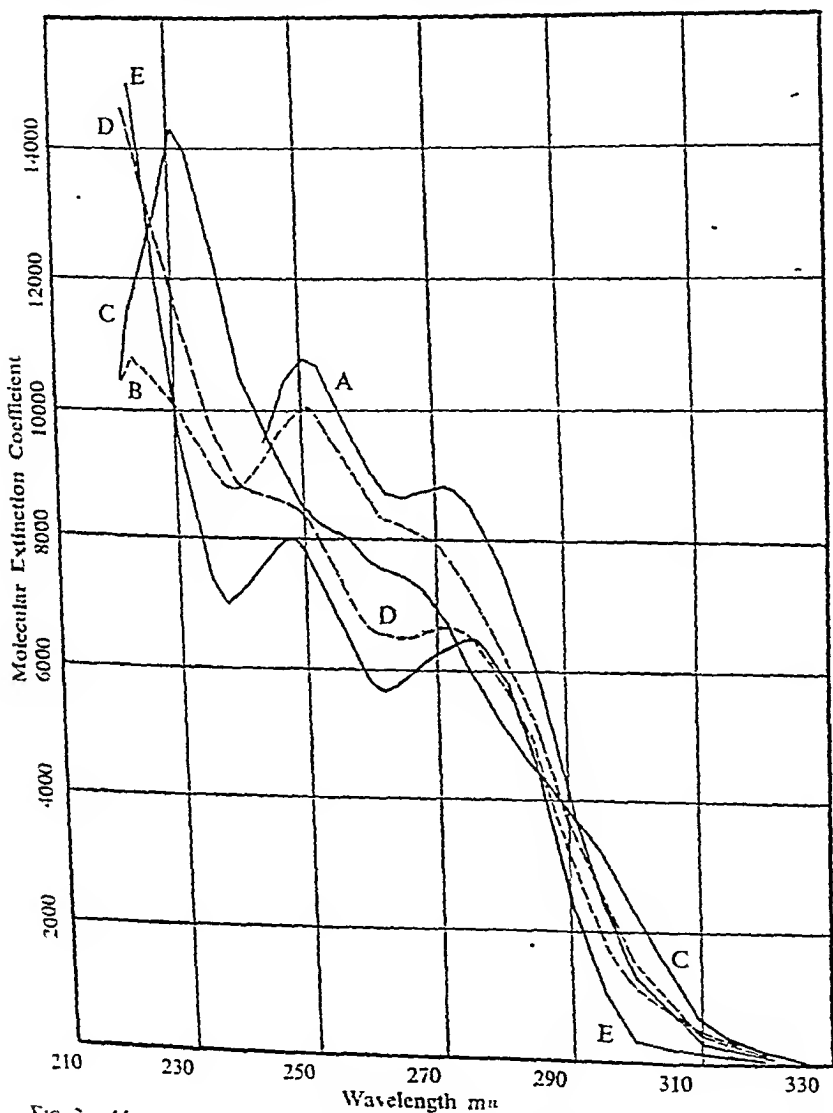


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break in the electrometric titration curve (Fig. 1). The spectrum of 2-thiouracil (Fig. 4) is in general agreement with the graphical results of Elion, Ide and Hitchings⁸ and of Miller, Roblin and Astwood³.

It was not possible directly to calculate the dissociation constants from absorption measurements (see e.g. Morton and Tipping⁹ for violuric acid, Stuckey¹⁰ for barbituric acid), as the relationship between absorption maxima and ionisation was not linear. It is obvious from this, and from

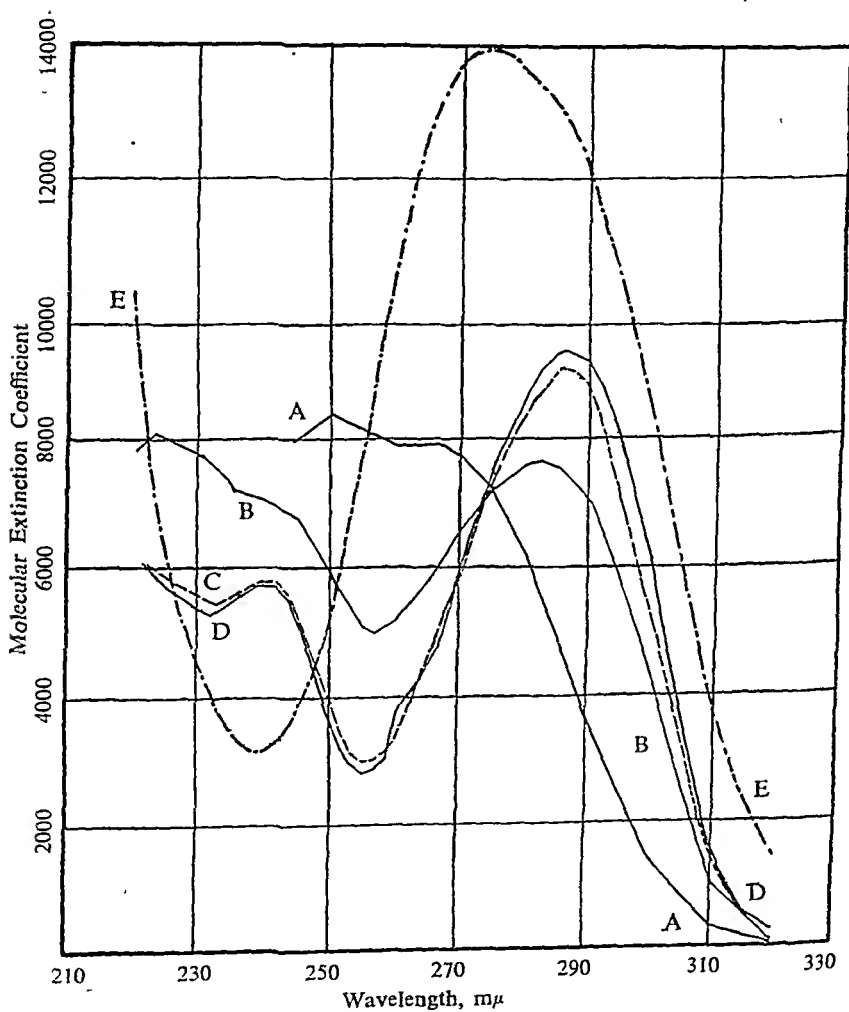


FIG. 4. Absorption spectra of 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine. A, at pH 0; B, at pH 1; C, at pH 2; D, at pH 12. E, 2-Thiouracil, at pH 7.

the fundamental spectrum changes, that variations in pH must be causing spectrum changes associated with resonance effects or with more than one group in the molecule.

ABSORPTION SPECTRA OF THIOURACIL DERIVATIVES

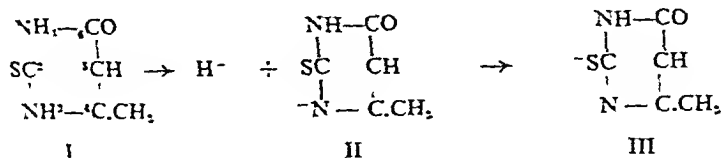
DISCUSSION

Tautomerism in 2-thio-4-methyluracil (I) can theoretically occur in more than one part of the molecule, notably involving the hydrogen attached to the nitrogen atom in the 3-position producing a $\Delta^{2,3}$ structure, or involving the hydrogen attached to the nitrogen atom in the 1-position producing either a $\Delta^{1,2}$ or a $\Delta^{1,6}$ structure. The potentiometric titration curve for this compound (Fig. 1) shows a single dissociating group with a pK_2 value in aqueous ethyl alcohol of 8.5, but the fact that the absorption

TABLE I

	pH	λ_{\max} m μ	ϵ_{\max}
2-Thiouracil	7	274	13,940
2-Thio-4-methyluracil	2	275	16,980
	7	275	16,160
	8	272	16,100
	9	267	11,800
	10	232.5	12,180
		260	11,500
		305	8,050
	12	232.5	11,070
		260	10,430
		305	7,080
2-Methyl-1-mercapto-4-methyl-6-oxypyrimidine	0	250	10,800
		270	8,810
	1	222.5	10,730
		250	10,030
	2	230	14,260
	8	272.5	6,630
	9	247.5	7,980
		275	5,960
1,4-Dimethyl-2-methyl-1-mercapto-6-oxypyrimidine	0	250	8,320
	1	222.5	8,040
		232.5	7,620
	2	241	5,750
		285	9,050
	12	241	5,740
		286	9,420

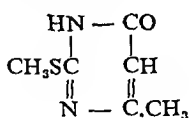
changes do not show a linear relationship between peak values and ionisation indicates that spectrum changes are not directly associated with a single ionic structure.



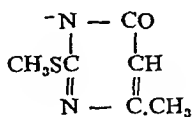
The absorption spectra of 2-thio-4-methyluracil show a main broad peak ca. 270 m μ with relatively little change at pH values up to 8. This peak corresponds to the undissociated form I, by analogy with other pseudo-acids, e.g., barbituric acid derivatives, the thio-ketone form being present in aqueous acid solution. It is noteworthy that Schneider and Halverstadt¹¹ found from a study of dipole moments in dioxan that 2-thiouracil possessed the structure corresponding to I. The change in

spectrum accompanying dissociation (from pH 7 to pH 10) is fundamental, with the splitting of the main band into three subsidiary bands; at pH 10 the change is virtually complete, the absorption maxima at higher pH values showing a slight decrease only, common to the spectra of many pseudo-acids in alkaline solution (cf. Stuckey¹⁰), the main characteristics being retained. The reason for the main spectrum change is not easily interpreted, but it is probable that this follows ionic rearrangement, predominantly with the production of structures II and III with associated resonance effects. This is supported by the fact that pH measurements show only one dissociating group. Any second dissociation with the addition of a further double link (from subsequent tautomerism) would produce the basic pyrimidine structure with three double links; this would have characteristically lower absorption values, which are not actually found. Methylation, with the production in the first instance of 2-methylmercapto-4-methyl-6-oxypyrimidine (IV), gives support to the supposition that the nitrogen in the 3-position is the dissociating group. The spectra of IV in alkaline solution show, broadly, a similar pattern to 2-thio-4-methyluracil in alkaline solution.

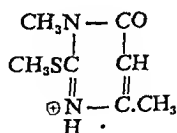
The spectrum of 2-thiouracil (Fig. 4) agreed with the graphical results of Elion, Ide and Hitchings⁸ and of Miller, Roblin and Astwood³. The peak values in acid solution were considerably lower than for the 4-methyl compound and lower than for 2-thiothymine. Although the introduction of a methyl group in the 4-position has no direct effect on the tautomerism it must obviously have some effect other than the normal weighting although exactly what is causing the increase in ϵ_{\max} is rather obscure. The parent uracil has ϵ_{\max} 11,000 at 258 $m\mu$. (Loofbourow, Stimson and Hart¹²) so that the replacement of oxygen by sulphur has caused only a slight increase in ϵ_{\max} .



IV



V



VI

2-Methylmercapto-4-methyl-6-oxypyrimidine (IV, Fig. 3) shows an absorption curve at pH 2 with a maximum at 230 $m\mu$, but lacking any peak values at longer wavelengths; at this pH , ionisation accompanied by the splitting off of a hydrogen atom does not take place to any extent and the spectrum will be that of the undissociated structure IV. There is a change in spectrum with the development of two subsidiary peaks in alkaline solution (pH 9) corresponding to dissociation as shown by the electrometric titration curve. The spectrum at pH 9 is due to the ion (V) with the possibility of resonance with a fully unsaturated pyrimidine ion having a 1:6 double link. The pH curve for 2-methylmercapto-4-methyl-6-oxypyrimidine shows a slight break with an inflexion corresponding to a radical change in the spectra *ca.* pH 1 and

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pH 0, undoubtedly due to the partial conversion of the tertiary nitrogen to a $=\text{NH}-$ structure.

1:4-Dimethyl-2-methylmercapto-6-oxypyrimidine shows no change from pH 2 to pH 12, which is to be expected in the absence of an ionisable hydrogen atom. The break in the pH curve between pH 0 to pH 1 corresponds, as with the monomethylated compound IV, to the addition of a proton to the tertiary nitrogen in the 3-position (VI).

The iodine absorption theory of anti-thyroid activity is supported by the work of Albert, Rawson, Merrill, Lennon and Riddell¹³, who found that the loss of thyrotropic activity occurring during exposure of a pituitary extract to iodine, was restored by treatment of the iodinated hormonal material with 2-thiouracil. The production of a disulphide compound incorporating the thiol form of the thiouracil molecule (III) in Astwood's iodination experiments, together with the findings of Williams and Kay³, suggest that any *mechanism* of iodine absorption depends on ionisation and subsequent ion tautomerism. In view of this it appears that ionisation is necessary for antithyroid activity in thiouracil derivatives. The present work has shown that 2-thio-4-methyluracil has a pK_a value in 50 per cent. aqueous alcohol of 8.5; in order to make this figure applicable to an aqueous solution it is necessary to apply a correction. Mizutani¹⁴ found values for ΔpK_a between water and 50 per cent. aqueous ethyl alcohol to be of the order of 0.9 to 1.2, with an approximate average of 1.1, for the numerous acids studied. It is unfortunate that a more precise correction cannot be applied since a small difference in pK_a in the region pK_a 6.6—8.0 corresponds to a big difference in percentage dissociation at pH 7.3; the very limited solubility of 2-thio-4-methyluracil, however, makes an accurate determination in aqueous solution a matter of difficulty. Assuming ΔpK_a to be 1.1 units, the corrected figure for 2-thio-4-methyluracil is, therefore, 7.4. This corresponds to a dissociation of 44 per cent. at a blood pH of 7.3. On the limited evidence available an appreciable dissociation of thiouracil derivatives at pH 7.3 would seem to be necessary for iodine absorption in anti-thyroid activity.

The peak absorption values in acid solution shown by 2-thio- and 2-thio-4-methyluracil provide a useful property for the analytical estimation of these compounds. This, together with the dissociation properties of thiouracil derivatives, is the subject of further investigation.

SUMMARY

1. The ultra-violet absorption spectra of 2-thiouracil, 2-thio-4-methyluracil, 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine and 2-methylmercapto-4-methyl-6-oxypyrimidine have been determined at varying pH values.

2. Electrometric titration curves have been plotted for 2-thio-4-methyluracil, 2-methylmercapto-4-methyl-6-oxypyrimidine and 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine, and pK_a values for these compounds in aqueous ethyl alcohol have been recorded.

3. The close connection between change in pH and change in ultra-violet light absorption for the compounds studied shows that absorption changes are due primarily to ionisation and subsequent ionic rearrangement.

4. On the basis of the iodine oxidation theory, the probable ionic nature of the iodine absorption mechanism suggests that thiouracil derivatives which are appreciably ionised at pH 7.3 are most likely to exhibit anti-thyroid activity.

The writer would like to thank Miss H. M. Oster for technical assistance and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

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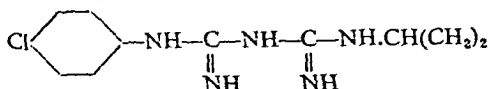
THE DETERMINATION OF PROGUANIL

BY H. E. STAGG

From the Analytical Laboratories, Imperial Chemical Industries, Ltd., Blackley, Manchester

Received March 31, 1949

PROGUANIL (Paludrine), introduced as an antimalarial drug in 1946, has the constitution N_1 -*p*-chlorophenyl- N_5 -isopropylbiguanide.



It is a strongly basic substance which is administered orally as tablets containing the monohydrochloride and by injection as a 5 per cent. solution of the lactate.

Methods for the determination of traces in biological fluids have been described by Spinks and Tottey¹ and Gage and Rose². Spinks and Tottey employ hydrolysis with $N/4$ hydrochloric acid in a sealed tube at 100°C . followed by diazotisation of the *p*-chloroaniline, coupling with N - β sulphatoethyl-*m*-toluidine and colorimetric determination of the azo dye which is formed. Gage and Rose's method depends on formation and extraction of the copper-complex $(\text{C}_{11}\text{H}_{15}\text{N}_5\text{Cl})_2\text{Cu}$ (see page 392) with benzene and determination of the copper in the extracted complex by the well-known dithiocarbamate method.

Neither method, however, is of sufficient accuracy to be satisfactory for the determination of major amounts, either in the bulk product or in pharmaceutical preparations, and the methods which have been developed for these purposes are described herein.

METHODS OF ANALYSIS

1. **VOLUMETRIC DETERMINATION IN THE HYDROCHLORIDE BY TITRATION WITH ACID** The drug is in reality a diacidic base, and although the form in which it is mainly encountered (the monohydrochloride) is neutral in aqueous solution, this salt can be dissolved in glacial acetic acid and the second basic group can be titrated with a standard solution of perchloric acid in glacial acetic acid in the manner described by Bandel and Blumrich for the titration of weak bases with strong acids³. The indicator is α -naphthol benzein, which changes from yellow to green at the end-point.

This method has the advantage of rapidity and the results are not affected by normal tablet excipients (e.g. starches, gums, sugars, etc.); it can, therefore, be used for the direct determination of the content of tablets

Reagents required: 1. Acetic acid, glacial, analytical reagent quality.

2. $N/10$ solution of perchloric acid in glacial acetic acid: dissolve the equivalent of 10.05 g. of perchloric acid (calculated from the acid content

3. The close connection between change in pH and change in ultra-violet light absorption for the compounds studied shows that absorption changes are due primarily to ionisation and subsequent ionic rearrangement.

4. On the basis of the iodine oxidation theory, the probable ionic nature of the iodine absorption mechanism suggests that thiouracil derivatives which are appreciably ionised at pH 7.3 are most likely to exhibit anti-thyroid activity.

The writer would like to thank Miss H. M. Oster for technical assistance and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

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DETERMINATION OF PROGUANIL

Reagent required. 5. Ammoniacal cupric chloride solution: dissolve 22.5 g. of cupric chloride, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, in 200 ml. of water and add 100 ml. of ammonia solution (880).

Determination. Weigh 0.5 to 0.7 g. of sample into a 250-ml. beaker, and dissolve in 50 ml. of water by gentle warming. Cool in an ice bath to 5° to 10°C . and add ammoniacal cupric chloride solution (reagent 5), with stirring, until the solution remains a deep blue colour. Allow to stand at room temperature for at least 1 hour and then filter on a sintered glass crucible, porosity 3—preferably one having a large filtering surface—fitted with an asbestos pad and previously dried at 130°C . to constant weight. Wash the precipitate with 100 ml. of dilute ammonia and then with cold water until the washings are quite colourless. Dry at 130°C . ($\pm 5^\circ$) to constant weight.

$$\frac{\text{Wt. of precipitate} \times 1.020}{\text{Wt. of sample}} = \text{per cent. calculated as hydrochloride, mol. wt. 290}$$

DISCUSSION

The colour change which occurs at the end-point of the volumetric method extends over a range of about 0.5 ml. of titrant in a titre of 25 ml., and the averages of duplicate tests by two operators upon the same sample often differ by 1.5 per cent. The method has, however, the advantage of rapidity and is, therefore, of special value where large numbers of determinations have to be carried out, as, for example, in the routine examination of tablets.

The precision of the gravimetric method is considerably higher than that of the volumetric method, and the averages of duplicate tests by two operators upon the same sample usually agree within 0.5 per cent. Recovery from specially purified hydrochloride has not been determined; this was not considered to be necessary, since the technical hydrochloride (i.e., the material which is normally manufactured for use in pharmaceutical preparations) is itself a very pure product, and the sum of the determined constituents—active agent, *p*-chloroaniline, moisture and ash—in technical samples is normally over 99.0 per cent.

Analysis of a typical sample of technical proguanil hydrochloride is given below:—

content, by gravimetric method. calc. as hydrochloride, mol. wt. 290	99.5 per cent.
<i>p</i> -Chloroaniline	Less than 0.01 per cent.
Moisture (by loss of weight on heating at 100°C .)	0.1 per cent.
Sulphated ash	0.05 per cent.
Total	99.6 per cent.

of the 60 per cent. aqueous solution) in 50 ml. of acetic acid; add this mixture, a drop at a time, to an amount of freshly distilled acetic anhydride (boiling point 137° to $139^{\circ}\text{C}.$) which is exactly sufficient to combine with the water present in the perchloric acid, cooling the mixing vessel in ice during the addition. Dilute with acetic acid to 1 litre.

3. Standard solution of sodium acetate in acetic acid: cautiously dissolve 1.325 g. of sodium carbonate, previously dried for 5 hours at $300^{\circ}\text{C}.$, in 50 ml. of acetic acid, transfer to a 250-ml. measuring flask and make up to volume with acetic acid.

4. α -Naphthol benzein indicator: dissolve 0.2 g. of α -naphthol benzein in 100 ml. of acetic acid.

Determination. Standardise the perchloric acid (reagent 2) by titrating 50.0 ml. of the standard solution of sodium acetate (reagent 3) and 1 ml. of the indicator (reagent 4) until the colour changes from brown to green. The temperature of the perchloric and sodium acetate solutions should be kept as near to $20^{\circ}\text{C}.$ as possible for all measurements of volume in order to minimise errors due to the large coefficient of expansion of acetic acid.

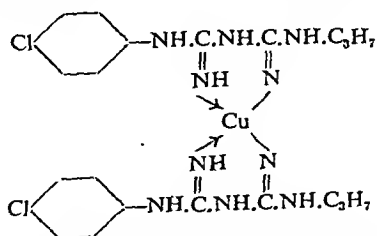
Experience has shown that the normality of this solution remains constant, under ordinary conditions of storage in a stoppered bottle, for at least 3 months.

Weigh about 0.8 g. of the sample, or its equivalent in tablet form, into a dry conical flask and dissolve in 30 ml. of acetic acid, warming to a temperature not exceeding $80^{\circ}\text{C}.$ Cool to room temperature, add 0.5 ml. of indicator solution and titrate with the perchloric acid solution until the colour of the titration matches that obtained in the standardisation.

If $B =$ vol. of $N/10$ perchloric acid required

$$\frac{2.90B}{\text{wt. of sample}} = \text{per cent. calculated as hydrochloride, mol. wt. 290}$$

2. GRAVIMETRIC DETERMINATION IN THE HYDROCHLORIDE OR LACTATE BY PRECIPITATION OF THE COPPER COMPLEX. When an excess of ammoniacal cupric chloride solution is added to a cold aqueous solution of the hydrochloride or lactate, the base is precipitated as a copper complex containing ten atoms of nitrogen for each atom of copper. There is little doubt that this precipitate is an inner complex having the formula:



and it has been found to be suitable for gravimetric determination, being easily washed free from the excess of reagent and dried at $130^{\circ}\text{C}.$

A NOTE ON THE CONVERSION OF ω -TRIBROMOQUINALDINE TO ω -DIBROMOQUINALDINE AND THE PRODUCTION OF QUINALDIC ALDEHYDE

By L. K. SHARP.

Received March 3, 1949.

ω -DIBROMOQUINALDINE cannot be prepared by selective bromination of quinaldine, the only product isolated being the ω -tribromo derivative, but the former may be prepared by reduction from the latter. Hammick¹ reduced tribromoquinaldine by means of stannous chloride in acetone solution, obtaining a yield of 60 per cent. of theory. In order to separate the product from tin compounds, steam distillation was employed. As dibromoquinaldine is only slightly volatile in steam the purification process is tedious. The reduction may however be performed by refluxing ω -tribromoquinaldine with a 20 per cent. v/v solution of sulphuric acid in alcohol and pouring the mixture into water. The product separates almost pure in excellent yield, and a single crystallisation from alcohol is all that is necessary. The identity of the product was confirmed by its conversion, by means of alcoholic silver nitrate, to quinaldic aldehyde by the method of Hammick².

EXPERIMENTAL.

ω -Tribromoquinaldine (10 g.) prepared by Hammick's³ method was refluxed for 4 hours with a mixture of alcohol (97 per cent.) (80 ml.) and concentrated sulphuric acid (20 ml.), and the mixture then poured into water. The white precipitate was washed and dried. Yield 7 g. (88 per cent. of theory). M.pt. 119°C. After recrystallisation from alcohol the pure product melted at 120°C. Found: C, 39.95; H, 2.67; N, 4.9; Br, 52.9 per cent.: $C_{10}H_7NBr_2$ requires C, 40.0; H, 2.3; N, 4.7; Br, 53.3 per cent.

PROOF OF CONSTITUTION.

2.5 g. in 15 ml. of boiling alcohol was treated with silver nitrate solution and the product worked up in the manner described by Hammick². After steam distillation 0.9 g. (70 per cent. of theory) of quinaldic aldehyde was obtained: M.pt. 69°C.; mixed melting-point with quinaldic aldehyde, 70°C.

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SUMMARY

1. Two methods are described for the determination of proguanil hydrochloride: one method depends upon titration with perchloric acid in glacial acetic acid solution, and the other upon gravimetric determination of the copper-complex precipitated from cold aqueous solution by ammoniacal cupric chloride.

2. The volumetric method is rapid and is applicable to the determination in tablets, but is less precise than the gravimetric method.

3. The gravimetric method is applicable also to the lactate.

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CEPHAËLIS IPECACUANHA

potash, magnesia and lime; a minimum temperature of 50°F. and a maximum of 100°F., with smaller variations in temperature during summer and winter and during day and night; and a minimum rainfall of about 90 in. distributed during monsoon, winter and spring.

The experiments reported here were chiefly concerned with germination of seeds, growth of cuttings and of young plants of *Cephaëlis Ipecacuanha* (Kew variety) obtained from Mungpoo under the changed tropical conditions of the plain. The experiments were made under thatched sheds and on prepared soil beds, as is usual in Mungpoo.

Germination from seeds. The seeds, which usually take 4 to 6 months to germinate, have a very hard coat, are plano-convex and 3 to 5 mm. in length. They were sown on prepared beds in December, 1946, and January, 1947. They were watered morning and evening. By the end of April, the December and January sowings started germination almost at the same time. The curved seedlings were very weak and took 15 to 20 days to become erect with the hard seed coat still enclosing the apices of the first pair of leaves. The seedlings were so delicate and the hard seed coats were so tough that it was not possible to remove the seed coats without damaging the seedlings. The seedlings died under these conditions. Later on monthly sowings of seeds and the treatment of the seeds with acids to remove the hard seed coat were tried. The seeds were also subjected to low temperatures for different periods and to alternate high and low temperatures before sowing. But in no case did the seeds sprout even after 6 or 7 months. The causes of dormancy are being studied in detail.

Growth from Cuttings. By the end of February, 1947, some young plants were obtained from Mungpoo. A few cuttings of roots, stems and leaves from two such young plants were made and planted on prepared beds. The cuttings were kept under humid conditions by a cover of wet moss. They were watered every 3 hours during the day with a suitable arrangement to avoid waterlogging. During April some of the root cuttings began to sprout, but the stem cuttings did not and the leaf cutting began to rot. With the approach of the monsoon (i.e., during the months of May, June, July, August and September) more of the root cuttings sprouted and showed progressive activity by the production of minute leaves and 3 to 4 nodes. The internode varied in length from 1 to 2 cm. The activity of the cuttings was best in August. By the beginning of November the root cuttings were affected for the time with browning of the apices of their leaves. Browning progressed with the advance of the month and resulted in gradual drying up of the cuttings.

Growth of young plants. A few of the young plants obtained from Mungpoo by the end of February, 1947, were planted on prepared beds in earthen pots. They were watered every three hours during the day. The leaves of young plants within a few days showed browning of their apices and periphery. Browning progressed from these areas to the middle region of each lamina. As a result, the leaves soon crumpled

A PRELIMINARY INVESTIGATION ON THE GROWTH OF *CEPHAËLIS IPECACUANHA* (BROT) A. RICH, UNDER TROPICAL CONDITIONS AT CALCUTTA

By GOPAL C. MITRA AND DIPTISH CHAKRABORTY

From the Bengal Immunity Research Institute, Calcutta

Received October 5, 1948

INTRODUCTORY

THE first plant of *Cephaëlis Ipecacuanha* was taken to Calcutta by Dr. King in 1866, and in about 1868 experiments with a view to the introduction of the plant into India were begun by Dr. Anderson in the Royal Botanic Gardens, Calcutta. The original stock of plants came from Kew and Edinburgh. Gammie observed that the varieties from Kew and Edinburgh differed greatly, the leaves of the former being more rough and hence more hardy than the latter. Balfour^{1,2} had previously described the difference in the character of the leaves between the plants received from Kew and those sent by Gunning from Rio de Janeiro. The leaves of the Kew plants are firmer in texture, somewhat coriaceous, elliptical or oval, with apex rather blunt, and margin wavy. The leaves of the Rio de Janeiro plants are thinner and more delicate in texture, the shape is rather elliptico-lanceolate, the apex pointed, and the margin less wavy; in the young state the leaves are fringed with hairs; the plants grow more freely and are less shrubby. The Kew plants were also characterised by flowers possessing long stamens and short style as described by Hooker³, while the plants produced from Rio de Janeiro specimens showed two forms of flowers, viz., one with a short style and long stamens as in the Kew plant and the other with a long style and short stamens. Under cultivation by Gammie the Edinburgh plants did not survive for more than a year or two, but plants of the Kew variety, in the shade, lived. The Kew variety thus seemed to be more hardy and more suitable for cultivation in India. Later ipecacuanha was successfully cultivated in the Sikkim Himalayas. The seeds and plants for the present investigation were obtained from a nursery at Labdah. Evidently the original stock of these plants is the Kew variety, and they resemble the description and drawings given by Bentley and Trimen⁴ and by Balfour². But to the authors it seems that the characters of the two forms as mentioned by Balfour have become less clearly defined during natural fertilisations at the early period of their acclimatisation in India. The present production of the drug in India is insufficient to meet the demand, and more attention and systematic research is needed to improve the quality and yield.

EXPERIMENTAL

All the preliminary experiments on the cultivation of *Cephaëlis Ipecacuanha* in India show that for favourable growth and development the plant requires:—a forest area with sandy loam soil rich in humus,

of Calcutta throughout the year, and in some months the difference is as great as 20°F., whilst the mean maximum temperature curve at Labdah is slightly below that of Calcutta, excepting in the months of July and August. So the mean maximum temperature of the plain may not affect the growth of the plants, but the duration of the maximum temperature in the hills and in the plains is to be taken into consideration. In the hills the mean maximum temperature lasts for a few hours, while in the plains it persists for a longer time. As regards the duration of the mean minimum temperature it is just the reverse. The rainfalls of the two places show wide differences. The total rainfall during a year at Labdah is 137 in., while at Calcutta it is 63 in.

It was seen from these experiments that the high temperature of the summer months, April, May and June, did not prevent the natural growth of the cuttings and young plants and the germination of seeds. Further, under natural conditions, the growth of the cuttings and of young plants improved with the onset of rains, and was found to be the best in August. In November, however, the cuttings and young plants showed the signs of decay as seen by the browning of their leaves. Again, it was found that on increasing the humidity, which was 64, by growing the cuttings and young plants under bell-jars their growth was revived to a great extent as seen by the development of the branches and leaves. Thus from November onwards, through the winter months, the condition of the growth of the cuttings and young plants, if kept in increased humid condition, was much better than what was found in the natural condition during the month of August. The humidity inside the bell-jar may be assumed to approximate to that prevailing in the monsoon, say, in the month of August. The observations indicate the favourable influence of the lower temperature of the winter months as compared with August, provided the humidity is not allowed to decrease, and the mean maximum temperature which persists for a longer duration in the plain appears to be above the optimum temperature, otherwise we would have expected the best growth in August.

The anatomical structure of the leaves also indicates that the plants are sensitive to desiccation, which was supported by the revival of growth of the cuttings and young plants in November when they were grown under the bell-jars in increased humid conditions. The browning of leaves in November can be ascribed to the desiccatory effect of lowering of humidity in that month.

SUMMARY -

1. Under the tropical conditions of the suburbs of Calcutta the seeds of *Cephaelis Ipecacuanha* (Kew variety) sprouted in April, 4 and 5 months after their sowing, but quickly died.
2. The young plants and cuttings, especially the roots, showed the greatest activity in August when the rainfall was 15.4 inches and the mean minimum temperature 79.5°F., whilst in November, when there

and were shed. Microscopical examination of the brown patches of the leaf lamina showed that they were not due to fungal attack, and the anatomical structure of the leaf showed the characteristic tissue development and differentiation of a shade and moisture-loving plant. Frequent spraying with water failed to check the browning of the leaves, and by the end of April all the leaves of the young plants were shed. Though they were completely defoliated, they were alive, as shown by the freshness of their terminal buds, and the gradual appearance of axillary buds gradually increased during the months of May, June, July, August and September. The buds only slightly expanded into one or two pairs of minute leaves. The growth was best in August. By the beginning of November the young plants were again affected with browning of the apices of the small leaves of terminal and axillary buds. Browning of the apices of leaves of the buds similarly progressed very quickly, and by the end of the third week of the month resulted in their drying up.

Experiments under scientifically increased humid conditions. Towards the end of the third week of November, when the root cuttings and the young plants were both showing definite signs of drying up, they were subjected to increased humidity for the purpose of studying its effect upon their growth. Accordingly, on November 21, 1947, the root cuttings and young plants, which were already affected with browning of their leaves, were covered with bell-jars leaving a little space for the access of air; inside each bell-jar a dish containing water was kept. Each morning and evening, fresh air was admitted so as to avoid the accumulation of carbon dioxide. Both root cuttings and young plants responded quickly to this treatment. It was observed that browning of apices of leaves of the root cuttings, which had already started, could not be checked, but, later, new leaves were formed. The effect was more marked in young plants. One of the axillary buds of a young plant near the base of its stem steadily increased in length and in the surface area of its leaves. At the beginning of the experiments the length of the axillary shoot was 1 cm., and 8 weeks later it had increased to 5 cm., while in the same period the length of one of the first pair of leaves of the axillary shoot which was 1.2 cm. and the other one which was 0.6 cm. had increased to 5.3 cm. and 3.1 cm. respectively. Two more pairs of leaves of the axillary shoot were developing by this time.

DISCUSSION

The preliminary experiments in the acclimatisation of plants in India show that the plants can easily be propagated from root cuttings and seeds, and that they prefer moist shady spots where there is much vegetable mould in the soil and an equable steamy atmosphere. The plants and cuttings, especially the roots, showed the best growth in August in nursery conditions but, in artificially increased humidity, during the winter months, the growth was much more than in August. The mean minimum temperature curve of Labdah is always far below that

THE PREPARATION OF INDONES

BY D. DALEV

Received September 25, 1948

THERE are many methods of preparing indones. One of the commonest is that which was given more or less at the same time by Löwenbein and Ulich¹ on the one hand, and by Weiss and Sauermann² on the other. This method is not very convenient because most of the indones produced by it contain much impurity in the form of 3-benzalpthalide and some by-products, and the mixture of the latter with the indones is a sticky, resinous substance which prevents the indones from crystallisation. Weiss and his fellow worker removed the benzalpthalide by saponification with concentrated alcoholic ammonia. Under such conditions the purification of the greater proportion of the indone is difficult. Weiss does not mention the reasons which made him choose ammonia for the saponification of benzalpthalide.

My experiments have shown that dilute aqueous solutions of alkaline hydroxides or carbonates do not change the indones to any marked extent when boiled. The latter do not change even when they are mixed with benzalpthalide. The crude indones which are produced, after they are distilled from the ether-benzene mixture at a temperature below 100° C., form a thick substance resembling molten resin. The by-products of the reaction, such as hydrocarbons and unchanged halogenides, are separated from this mass by steam distillation.

The use of steam has this advantage, that it continuously stirs the resinous substance and almost completely separates the volatile compounds. From this it appeared that the benzalpthalide in the crude indone would be easily soluble under these conditions if the medium was alkaline. Experiment proved that a 1 to 2 per cent. solution of sodium hydroxide is suitable for saponification. An aqueous solution of sodium or potassium carbonate can be used equally successfully. What quantity of unchanged benzalpthalide there is with the different indones is, of course, not known. It can safely be assumed that the hydroxide or the carbonate can be calculated for at least 50 per cent. of unchanged benzalpthalide. The saponification is completed in most cases in about half an hour.

EXPERIMENTAL

General method. The organomagnesium compound is produced from 0.1 g. mol. of halogenide with a corresponding excess according to Gilman's table³ in order to produce a 100 per cent. yield. The ether solution of the organomagnesium compound is transferred to a separating funnel in such a way as to prevent oxidation from the air. The flask is closed with a rubber stopper through which passes a short glass tube. The flask is turned over quickly, the tube is put in the separating funnel and almost all the ether solution is poured out of the flask in which there always remains a little of the ether solution of the organomagnesium com-

was no rain and the mean minimum temperature was 64.6°F., they showed signs of drying up.

3. Artificial increase of humidity at this stage revived the growth of young plants and cuttings. Onwards through the winter months the growth of the cuttings and young plants was maintained.

4. The observations indicate the favourable influence of the lower temperature of the winter months provided that the humidity is not allowed to decrease. The monthly mean maximum temperature of the plains appears to be above the optimum for growth of the plant.

This work has been carried out under the direction of Mr. S. N. Bal, Director, Pharmacognosy Laboratory, Government of India. The authors are grateful to Mr. S. C. Sen for providing plant material.

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PREPARATION OF INDONES

This method can be applied for the preparation of many different indones, and the work is being continued with a view to the producing triketones from which to prepare anthracene derivatives similar to those used as carcinogenics in cancer research.

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THE SPECTROPHOTOMETRIC DETERMINATION OF RUTIN AND QUERCETIN IN MIXTURES

By R. V. SWANN

This Journal, 1949, 1, 323-329

Corrections

Page 324, figure 1, horizontal scale, for 520 read 250.

Page 328, figure 2, horizontal scale, for 367 read 347.

vertical scale, for 0, 10, 20 per cent., etc., read 0.0,
1.0, 2.0, etc.

Bottom line, for Porter *et al.*² read Porter *et al.*¹.

pound and a little magnesium. A solution of 22.2 g. of benzalphthalide in 100 ml. of dry benzene is quickly poured in, followed by the solution, first drop by drop, and then in a thin stream, at the same time continually shaking the flask. Very often the inside of the flask becomes covered with a thick yellow-brown precipitate which fills the whole flask before all the ether solution is added. The temperature rises and the solution in the flask begins to boil. Boiling is prevented by cooling with water from time to time. After adding all the ether solution, the contents are stirred well with a metal rod and boiled over steam for 1 hour. At the bottom of the flask there almost always gathers a dark red sticky substance which is scraped off and stirred with a rod, two or three times while the heating lasts. It is left overnight and decomposed with ice and dilute sulphuric acid. In some cases water followed by sulphuric acid may be used—the temperature does not rise very much. Decomposition is completed in a short time, and the ether-benzene layer, of a colour something between orange and red, is separated. It is washed several times with a dilute alkali and with water and distilled directly in steam. At first the ether and the benzene distil quickly, and afterwards the unchanged halogenide and the other by-products of the reaction which are volatile in steam. The distillation in steam is continued until a clear liquid passes, sometimes over 10 or 12 hours. Distillation is then stopped and, without cooling, a sufficient quantity of dilute sodium hydroxide solution to produce about a 2 per cent. solution is poured in and distillation in steam is continued for another hour. When the resinous substance has deposited, the deep-red aqueous layer is poured off, and the resinous substance is again distilled in steam for another hour. When treated with alkali the indone often turns into a solid mass, and its removal from the flask becomes impossible. The solid mass contains a good deal of water, and for this reason its solubility in ether is very small, but it dissolves easily in chloroform. The chloroform solution is washed with water, and dried with dehydrated sodium sulphate and the chloroform is distilled off.

The following 12 indones have been prepared by this method and identified:—

	m.pt.
2:3-diphenylindone ⁴	153°C.
2-phenyl-3-tolylindone ⁵	123° to 125°C.
2-phenyl-3- <i>m</i> -tolylindone	113°C.
2-phenyl-3- <i>p</i> -tolylindone ⁷	133 to 134°C.
2-phenyl-3- <i>p</i> -bromo-phenylindone ⁷	172 to 174°C.
2-phenyl-3- α -naphthylindone	182 to 183°C.
2-phenyl-3-benzylindone	135°C.
2-phenyl-3- β -naphthylindone	132°C.
2-phenyl-3-cyclohexylindone	162 to 163°C.
2-phenyl-3-isopropylindone	110°C.
2-phenyl-3-isoamylindone	74°C.
2-phenyl-3-ethylindone	98°C.

of "surface moisture" only, when the amount present is small and the material is non-hygroscopic in character, 2 to 4 hours drying time was usually sufficient; (2) for the removal of "surface moisture" from materials of a hygroscopic nature, overnight drying (16 to 24 hours) may be required; (3) drying over sulphuric acid for removal of water of hydration was generally unsatisfactory, either because of incomplete dehydration or because of the time involved. It is recommended that substances dried at normal pressure and high temperatures should, in many cases, be dried at 105°C. rather than at 100°C. With drugs such as acacia and sterculia gum, the particle size was found to influence the loss on drying to a considerable extent, indicating that definite specifications for particle size as well as for temperature and time of drying were necessary; thus losses ranged from 10.2 to 14.4 per cent. on the same sample when reduced to different particle sizes. In vacuum drying, the working pressure is important, since greater losses are obtained with a low vacuum. It is recommended that vacuum drying should be carried out at a pressure of less than 5 mm. Hg. Tables are given covering over 1,000 recommended drying conditions: for U.S.P. XIII drugs; for the methods of drying applicable in general tests and analytical processes; and for the methods of drying applicable to U.S.P. XIII reagents. Methods of drying are similarly given for drugs, processes, and reagents of the N.F. VIII, following the principles outlined above. Graphs are included, showing the loss on drying with time of acetophenetidin, cotarnine chloride, emetine hydrochloride, sulphathiazole, digitalis, stibophen, ovary, and liquid glucose, under varying conditions.

R. E. S.

Morphine, Ammoniacal Zinc Ferrocyanide Solution as Reagent for. G. Denigès. (*Bull. Soc. Pharm. Bordeaux*, 1947, 85, 29) The reduction of ferricyanide by morphine, with subsequent formation of Prussian Blue, forms a familiar test for the alkaloid. Owing to the strong colour of ferric ferricyanide, the reaction is not very sensitive and cannot be employed quantitatively. These objections are removed by employing zinc in place of iron to detect the ferrocyanide formed. The reagent is prepared by mixing immediately before use, equal volumes of a 6 per cent. solution of potassium ferricyanide (free from ferrocyanide) and of a solution of 2.5 g. of zinc sulphate in 50 ml. of water to which is added 20 ml. of ammonia. A few drops of this reagent is added to about 1 ml. of the solution to be tested, when in presence of morphine a turbidity or precipitate appears in a short time, and may be estimated nephelometrically. The method may be applied to any clear preparation of morphine, even if coloured.

G. M.

Oxydimorphine in presence of Morphine, Determination of. N. Thörn and A. Ågren. (*Svensk. farm. Tidskr.*, 1949, 53, 33, 49.) Both morphine and oxydimorphine give colours with aromatic aldehydes in presence of sulphuric acid, but in the former case the extinction is very small at 600 μ . The method described below may be used for the determination of oxydimorphine formed in solutions of morphine after storage or sterilisation. For the test, 0.50 ml. of the solution to be tested is transferred to a dry test tube and treated with 10.0 ml. of a solution of 1.0 g. of vanillin in 100 ml. of sulphuric acid (95 \pm 1 per cent.) The reagent must be added slowly from a pipette, with continuous shaking and cooling. The mixture is stirred with a glass rod, immersed in a water-bath for 20 minutes, and then cooled in running water. A blank test is carried out at the same time using a solution of pure morphine of the same concentration. The difference in the extinctions is measured at 600 m μ . The official solvent of

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Boric Acid, Estimation of Minute Amounts of. N. Trinder. (*Analyst*, 1948, 73, 494.) Conditions have been worked out for the determination of small amounts of boric acid using the dyes alizarin blue S and the unsulphonated dye base of Solway purple (Colour Index No. 1073). Alizarin blue S in strong sulphuric acid changed in colour from purple to brown to green. The final concentration of sulphuric acid for this dye is important, a high acidity being needed. Alizarin blue S is only half as sensitive as the base of Solway purple but it has the advantage that it is stable in sulphuric acid solution and, except for fluorides and nitrates, is unaffected by the presence of large amounts of impurities. The base of Solway purple gives a colour change from pale yellowish-green to deep blue. It has the disadvantage that it sulphonates slowly to a blue compound at high sulphuric acid concentrations and gives a slight colour change if comparatively large amounts of manganese are present. It has the advantage that it is twice as sensitive as alizarin blue S and the dye colour deepens progressively over a wide range of boric acid concentrations. The most suitable dye for use in any particular determination will depend on the impurities present and the concentration of borate. Details are given of the methods using both dyes, the colour estimations being made using a Spekker absorptiometer with Ilford violet filters (No. 601) for alizarin blue S and Ilford Yellow filters (No. 606) for the unsulphonated dye base of Solway purple.

R. E. S.

Drying Conditions. A Study of U.S.P. XIII and N.F. VIII. N. L. Deahl, J. L. Powers and M. W. Green. (*Bull. Nat. Form. Comm.*, 1948, 16, 153.) The results of investigations on the drying conditions of over 1,000 substances contained in the U.S.P. XIII and N.F. VIII are given. Attempts were made in the work to standardise on a few different temperatures and conditions which were consistent with accurate and reproducible results. Another object was to establish the temperature and the time required for the drying of a large class of substances which are at present dried to "constant weight." Where substances gave identical results when dried under a wide range of conditions, the easiest and most acceptable laboratory procedure was recommended; weighings were made to within ± 0.1 mg., weighing bottles after removal from the ovens were cooled in desiccators over calcium chloride until they reached room temperature (kept constant at 25°C.); drying over sulphuric acid was done at 25°C. and vacuum drying was carried out at a pressure of less than 1 mm. Hg.; the temperature of drying was controlled to within $\pm 1^\circ\text{C.}$ of the desired temperature; other precautions taken followed the directions of the U.S.P. XIII; dryings were carried out on two or more samples of each substance. In general drying was carried out wherever possible under "standard conditions" as follows: (1) at 60°C.; (2) at 80°C.; (3) at 105°C.; (4) at 120°C.; (5) at 150°C.; (6) over sulphuric acid at room temperature; (7) in vacuum. The following general results are recommended for drying over sulphuric acid: (1) for the removal

Bacitracin, the antibiotic produced by the growth of the "Tracy I" strain of *Bacillus subtilis* was studied to ascertain its stability in various pharmaceutical preparations. It was found to parallel penicillin quite closely in its stability, or lack of it, with various substances. The dry substance was quite stable at 37°C., but showed definite decomposition at 56°C. At 80°C. decomposition occurred within 48 hours. Within a pH range of 5 to 7, aqueous solutions of bacitracin, with or without buffers, were stable for several months at refrigerator temperature, but lost about 50 per cent. of their activity in a week at room temperatures. Ointments prepared with anhydrous fatty bases showed good stability at room temperature, but attempts to prepare a stable water-miscible ointment were unsuccessful. A lozenge and a powder containing ephedrine for the preparation of nasal solutions were shown to be stable. Assay was by the cylinder-plate method using *Staphylococcus aureus* or *Micrococcus flavus*. G. R. K.

Lactobacillus casei, Growth Factors for. F. W. Chattaway, D. E. Dolby and F. C. Happold. (*Biochem. J.*, 1948, 43, 567.) The concentration and separation from liver of further factors promoting acid production by *Lactobacillus casei* are described. Importance is attached to the preparation of the casein digest used in the growth medium as some processes do not remove appreciable *L. casei* activity. Growth factors insoluble in saturated baryta were separated from the crude liver extract together with at least three factors soluble in saturated baryta and in silver nitrate at pH 1.0; these consist of a filtrate fraction which is not adsorbed on alumina at pH 3. and two fractions which are adsorbed, one of which is eluted with a 20 per cent. v/v solution of methyl alcohol or water and the other with 0.5 per cent. w/v solution of ammonia. The three latter components have properties dissimilar to both pteroyl glutamic acid and the folic acid of Mitchell *et al.* (1941). The greatest concentration of folic acid-like material was found in the silver salts insoluble at pH 1 (from the baryta-soluble material) which contained none of the components discussed above. The differential action of ninhydrin and nitrous acid, and of esterification and acetylation, upon the activity of the material eluted by a 2 per cent. w/v solution of ammonia for *Streptococcus faecalis* R. and *L. casei*, confirms that there are two components present in this material. The fact that the above chemical treatment affects the filtrate fraction and the baryta-insoluble fraction dissimilarly to one another, and to the above ammonia-eluate material, is evidence that four separate and distinct factors are present. R. E. S.

Penicillin Activity *in vitro*, Enhancement by Vitamin K₅. Robertson Pratt, J. Dufrenoy and P. P. T. Sah. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 38, 435.) The addition of vitamin K₅ (2-methyl-4-amino-1-naphthol hydrochloride) in concentrations of 0.1 to 10 mg./l. enhances the effect of penicillin against *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli* as measured by the cup plate method to a greater extent than would be expected from the sum of the activities of the two components acting separately. The magnitude of the enhancement depended on the concentration of K₅ and of penicillin and on the test organism. The enhancement is particularly marked with *E. coli*. A. L.

Penicillin, New Absorption Delaying Vehicle. F. H. Buckwalter and H. L. Dickison. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 472.)

ABSTRACTS

the Swedish Pharmacopœia for morphine injection contains 5 per cent. of glycerol and 15 per cent. of alcohol, and the method may be applied directly to such solutions, but the extinction is somewhat different, as shown by the table below.

Oxydimorphine in solution per cent.	Extinction	
	in water	in dilute glycerol-alcohol
0.2	0.190	—
0.4	0.372	0.315, 0.325
0.6	0.530, 0.549	0.456, 0.463
1.0	0.894, 0.907	0.814, 0.844

G. M.

Procaine Penicillin G, Spectrophotometric Determination of Procaine in. C. V. St. John. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 343.) Weigh accurately approximately 50 mg. of procaine penicillin G and transfer to a 100 ml. volumetric flask. Dilute to volume with distilled water and shake until completely dissolved. Transfer 5 ml. of this dilution to a 250 ml. volumetric flask, dilute to volume, and measure the optical density of the resulting solution at 290 μ against water in the reference cell. Obtain the concentration of the solution by calculation from an extinction coefficient determined on a sample of pure procaine hydrochloride or a sample of pure procaine penicillin G analysed by the chloroform shake-out titrimetric procedure. The accuracy of the method compares very favourably with that of the conventional method. Care must be taken, however, to clean the cells frequently with dichromate-sulphuric acid cleaning solution and to make careful adjustment of the instrument. Cell calibration should also be checked. The arithmetical average of the readings for several adjustments of the instrument on each of two fillings of the cell should be taken.

S. L. W.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Anti-Pernicious Anæmia Factor, Presence of Cobalt in. E. Lester Smith. (*Nature*, 1948, 162, 144.) The use of the borax bead test and the specific red colour reaction with nitroso R salt reveals the presence of cobalt in the ash of the anti-pernicious anæmia factor recently isolated as red needle-shaped crystals. The crystals, dried in vacuo at 56°C. contain 4.0 per cent. of cobalt, estimated colorimetrically with α -nitroso- β -naphthol. Assuming 8 per cent. loss on drying and one atom of cobalt per molecule, this corresponds to a molecular weight of 1,600, in agreement with the result (1,500 to 1,750) obtained by X-ray crystallography. The different molecular weight (3,000) found by diffusion may be due to errors in the method, impure material, or possibly association in solution. The molecule also contains three atoms of phosphorus. American workers have confirmed the presence of cobalt and phosphorus in vitamin B₁₂ isolated by them.

G. B.

Bacitracin, Stability of. G. C. Bond, R. E. Himelick and L. H. Macdonald. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 30.)

obtained which gave $[\alpha]_D^{26} - 86.1^\circ$ (1 per cent. in water) and $C_{27}H_{49}N_7O_{17} \cdot 3HCl$. When assayed with *Klebsiella pneumoniae* in a broth-dilution test, the trihydrochloride dihydrate had a potency of 820 units/mg. and on this basis the anhydrous material would have an activity of 891 units/mg. The trihydrochloride of mannosidostreptomycin crystallised in the form of isotropic hexagonal plates. By means of the counter-current distribution method, this material was also shown to be a single entity and to be free of streptomycin. Drying at $55^\circ C.$ *in vacuo* gave $C_{27}H_{49}N_7O_{17} \cdot 3HCl \cdot 2H_2O$; at $100^\circ C.$ *in vacuo* analysis showed $C_{27}H_{49}N_7O_{17} \cdot 3HCl$, $[\alpha]_D^{26} - 54.1^\circ$ (1 per cent. in water). When assayed with *K. pneumoniae* in a broth-dilution test, the anhydrous mannosidostreptomycin had a potency of ca. 210 units/mg.

R. E. S.

Vitamin A in Whale-Liver Oil, Chromatographic Estimation of. N. T. Grigman, G. P. Gibson and J. P. Savage. (*Analyst*, 1948, 73, 662.) The method given is based on the fact that the main components of the unsaponifiable fraction of whale-liver oil are selectively adsorbed on weakly active alumina in the order: anhydro-vitamin A < oxidised vitamin A < vitamin A (alcohol) < kitol < sterols < selachyl alcohol. The technique consisted of depositing the material on the column from a non-polar solvent and then developing and eluting the chromatogram with solvents of progressively increasing polarity; the eluate was collected fractionally and Carr-Price spot tests were used to identify the vitamin A portions, from which aliquots were bulked for spectrophotometric estimations. A quantity (approximately 0.35 to 0.40 g.) of the unsaponifiable fraction in light petroleum was used for chromatography and 25 ml. quantities of light petroleum-ether mixture were used to elute the fractions, the proportion of ether being gradually increased in each succeeding fraction. The eluate was collected in 5 ml. fractions and a few drops of solution from individual tubes were tested with Carr-Price reagent to establish the range of tubes containing the vitamin. The tubes corresponding to the zone below the vitamin A usually give a reddish-purple colour with the reagent; while those corresponding to the zone above the vitamin gave a bluish-purple or greenish-purple colour. Both these colours are readily distinguished from the bright blue of the vitamin A solution. Moreover, in a good chromatogram, the set of vitamin A tubes will be separated at either end from the sets containing the adjacent zones by one of two tubes whose eluate content is almost nil; these correspond to the inter-zone regions in the chromatogram and will give only a faint coloration with the Carr-Price reagent. Aliquots drawn from those tubes showing vitamin A reaction were pooled, diluted with cyclohexanol and $E_{1\text{ cm}}^{1\text{ per cent}}$ 326 to 328μ was measured; if the fraction is pure, the maximum of the absorption curve will lie between 326μ and 328μ , $E_{300\mu}/E_{\lambda_{\text{max}}}$ will be not more than 0.63, and $E_{360\mu}/\lambda_{\text{max}}$ not more than 0.35. Details are given of the full analysis by this method of a sample of whale-liver oil. The method could be extended to more normal oils. Chromatography of the unsaponifiable matter of a shark liver oil, a mixed fish-liver oil diluted in vegetable oil, a distilled vitamin A ester concentrate, and a cod-liver oil, showed that of the total absorptions at 326μ (on the whole oil for the first three samples and "via unsap." for the cod-liver oil) the following fractions were due to vitamin A: 85, 92, 91 and 90 per cent., respectively. Recovery experiments on vitamin A acetate dissolved in vegetable oil and in dolphin oil gave results of 97 to 99 per cent. on the unsaponifiable fractions.

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Streptomycin, Degradative Studies on. M. L. Wolfrom and W. J. Polglase. (*J. Amer. chem. Soc.*, 1948, 70, 2835.) An inactive product was obtained by degradation of dihydrostreptomycin; this, when acetylated with pyridine and acetic anhydride gave crystalline $C_{16}H_{24}O_{12}N_3 \cdot (CH_3C)(COCH_3)_{10} \cdot (I)$ m.pt. 261.5° to 262.5°C. $[\alpha]_D^{23} - 84$ (c, 1; water). Methanolysis of (I), designated deca-acetyldideguanyldihydrostreptomycin with subsequent reacetylation yielded hexa-acetylstreptamine, transition point 250°C. m.pt. 341 to 345°C., and methyl penta-acetyldihydro- α -L-streptobiosamide, m.pt. 194 to 195°C., unchanged on admixture with a specimen prepared from dihydrostreptomycin trihydrochloride, $[\alpha]_D^{23} - 120^\circ$ (c, 0.5; chloroform). (I) was found to be readily soluble in methyl alcohol, water and hot ethyl alcohol, sparingly so in chloroform, ethyl acetate and ethyl alcohol, and insoluble in benzene and ethyl ether. Aqueous solutions of N,N,N-tetra-acetyldideguanyldihydrostreptomycin (II), N²,N³-diacetyl streptamine (III) and N-acetyldihydro- α -L-streptobiosaminide were prepared by partial deacetylation of the aforementioned acetyl derivatives with 0.05N sodium hydroxide in water-dioxane. Treatment with periodate showed the presence in II of an α -glycol which is not in III, and this α -glycol is present in the streptamine moiety of II. The presence of such a glycol group indicates that streptobiosamine is attached at C4 of streptidine, thus confirming the result of Folkers. R. E. S.

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containing inactivated materials interfere and concentrations of penicillin X in excess of 1 per cent. will invalidate the results. Infra-red analysis of the individual penicillins is theoretically the ideal method, as the infra-red spectrum of any substance is unique. Unfortunately serious practical difficulties arise; the penicillin may contain impurities or inactivated materials which have absorption bands in the same position as the bands used for analysis, the penicillin may also be in the form of a salt which is insoluble in all the solvents that are of use in infra-red measurements, and degradation may occur when converting it to the free acid and transferring to a suitable dry solvent. The effect of crystal structure is not known and a separate calibration may be necessary with amorphous material, and it is not easy to measure I_0 , the intensity of incident radiation. A suitable internal standard, generally *dl*-alanine, in known concentration has to be mixed with the penicillin. The ratio $R = \log(I_0/I)_{703} \log(I_0/I)_{851}$ is determined for penicillin G, where I_0 and I are the intensities of incident and transmitted radiations respectively at both 703 cm^{-1} and 851 cm^{-1} . A calibration curve R against percentage of penicillin G is plotted by diluting the pure penicillin G with magnesium oxide; results using this curve have an accuracy of ± 2 per cent. For other penicillins different bands are used.

L. H. P.

Salicylate in Blood, Fluorophotometric Method for the Estimation of. A. Saltzman. (*J. biol. Chem.*, 1948, 174, 399.) One ml. of oxalated or citrated plasma is shaken in a test-tube with 9 ml. of a freshly prepared mixture of 1 volume of a 10 per cent. solution of sodium tungstate and 8 volumes of $N/12$ sulphuric acid, and the precipitated proteins are filtered off after 10 minutes. To 5 ml. of the filtrate 7 ml. of sodium hydroxide solution (40 per cent.) is added and the mixture is placed in a fluorophotometer. The fluorescence is directly measured within 30 minutes using the same filters as in the vitamin B_1 determination. The values are read off a standard reference curve, plotted by adding varying amounts of a standard salicylate solution, containing 1.16 mg. of sodium salicylate in 12 ml. of water to a reagent blank mixture, consisting of 5 ml. of tungstic acid and 7 ml. of sodium hydroxide solution (40 per cent.). These values must be multiplied by 2 to correct for the dilution. Salicylate concentrations of 1 to 2 mg. in 100 ml. are detectable, and both free and combined salicylate is determined. A modification of the ethylene dichloride method for determining salicylate concentration in the blood is also described.

L. H. P.

Streptomycin B, Chemical Assay of. W. B. Emery and A. D. Walker. (*Nature*, 1948, 162, 525.) The use of 0.2 per cent. anthrone, a reduction product of anthraquinone, in 95 per cent. sulphuric acid is described for distinguishing streptomycin B (a mannose) from streptomycin A; it can also be used for estimating the former in a mixture. The results obtained are in accord with those calculated from biological and chemical assays, making the accepted assumption about the relative biological activities of the two streptomycins. The glucosamine moiety, present in both molecules, does not react with the reagent.

R. E. S.

Urea in Blood, An Improved Diacetyl Reaction for the Estimation of. V. R. Wheatley. (*Biochem. J.*, 1948, 43, 420.) The enhancing effect of a number of substances on the diacetyl-urea reaction has been studied. Phenols were unsatisfactory and produced precipitates; with aromatic amines the colour produced was orange or red, and not yellow, whilst diphenylamine and its derivatives produced an intense magenta colour. The reaction with

BIOCHEMICAL ANALYSIS

Folic Acid, Polarographic Determination of. W. J. Mader and H. A. Frediani. (*Anal. Chem.*, 1948, 20, 1199.) The determination of folic acid can be made quantitatively and rapidly. The sample is dissolved in 1 per cent. tetramethylammonium hydroxide solution containing cadmium chloride as internal standard with sufficient ammonium chloride to prevent precipitation of the cadmium from alkaline solution. A polarogram of this solution yields two clearly defined waves, one at 0.74 volt (against the saturated calomel electrode) for the cadmium, and one at 0.98 for the folic acid. With known folic acid concentrations (and fixed cadmium concentration) a straight line results on plotting the step-height ratios of cadmium-folic acid against folic acid concentration using log log coordinates. Replicate runs on a fixed sample indicated a reproducibility of ± 2 per cent. if the drop rate was controlled within 0.7 second, and the temperature within $10^{\circ}\text{C}.$, variations which exceed the conditions ordinarily encountered in an analytical laboratory. The diffusion current constant as defined by Lingane was calculated to be 1.72. It was found more convenient to utilise the ratio-concentration curve and thus to read off sample concentrations directly in mg./ml. than to calculate sample concentrations. The method could be applied to folic acid tablets and to tablets with vitamin B₉ added, but could not be used in the presence of iron.

R. E. S.

Penicillin, Spectroscopic Estimation of. G. H. Twigg. (*Analyst*, 1948, 73, 211.) The author has reviewed various applications of ultra-violet and infra-red spectroscopy for the estimation of total and individual penicillins, and has discussed the limitations of these methods. The aim of spectroscopy is to discover in the absorption spectrum a band which is characteristic of each penicillin molecule as a whole. This is not attained in practice and the absorptions arising from separate parts of the molecule have to be used. Such a procedure may provide an estimate of total penicillin, but it leads to fundamental difficulties in assaying individual penicillins; impurities and deactivated penicillin products may contain similar molecular groupings and thus have bands almost identical with those of the penicillins. It is, therefore, likely that spectroscopic estimation of individual penicillins can only be applied to the pure material. The ultra-violet analysis of total penicillin depends on the development of an absorption band at 3220 \AA when an aqueous solution of penicillin is treated with acid under standard conditions. The band, which is due to an intermediate product, disappears after a time and the reading must be taken at its maximum intensity. The method can be used with impure material and gives an accuracy to within 5 per cent.; chemical methods of estimation are probably more speedy and accurate. The ultra-violet analysis of penicillin G depends on the development of absorption bands in the ultra-violet spectrum due to the phenyl group. Two methods have been developed. One compares the ultra-violet spectrum of the unknown sample with that of a known standard by photography and for pure samples of penicillin G gives an accuracy of ± 2 per cent. The second method is based on the relative absorption at 2630 \AA and 2800 \AA . Penicillin G has no absorption band at the higher wave-length but impurities and decomposition products have. All measurements of optical density are made with solutions of constant penicillin content (1.8 mg. per ml.) as both penicillin K and F show some absorption at 2630 \AA . The optical density difference $E_{2630} - E_{2800}$ is plotted against percentage of penicillin G content and gives a straight line, the analyses being evaluated from this calibration curve. Both these methods suffer from similar defects, phenyl-

were prepared and tested for curare-like activity on the phrenic-nerve diaphragm preparation of the rat (n = number of carbon atoms in the polymethylene chain):—*bis*-trimethylammonium series, n = 2, 3, 4, 5, 7, 8, 9, 10, 11, 12 and 13; *bis*-triethylammonium series, n = 2, 3, 4, 5, 7, 8, 9, 10 and 13; *bis*-strychninium series, n = 2, 3 and 5; *bis*-quinolinium series, n = 3, 5 and 10; *bis*-(phenyldimethylammonium) series, n = 3 and 5. In the *bis*-trimethyl series, the salt with n = 2 is about twice as active as tetramethylammonium iodide: salts with n = 3, 4 or 5 are only feebly active: activity increases from n = 7 to n = 9; salts with n = 9, 10, 11 and 12 are about 5 to 6 times as active as tetramethylammonium. In the *bis*-triethyl series, salts with n = 2 or 3 are relatively inactive; activity increases from n = 4 to n = 13. None of the members of the other three series was so active as the most active members of the *bis*-trimethyl series. In the rabbit head-drop test the *bis*-trimethyl member with n = 9 was nearly as active as tubocurarine chloride: the member with n = 10 was about 3 times as active. The *bis*-triethyl member with n = 13 was about two-fifths as active as tubocurarine. Some *bis*-onium salts augment the response of the rat diaphragm to maximum stimuli and inhibit the cholinesterase of caudate nucleus (dog). The sensitivity of the rat diaphragm to *bis*-onium salts differs greatly from that of the rabbit, and the rat diaphragm is less sensitive to methylstrychninium and more sensitive to tetramethylammonium iodide than the frog's sartorius, suggesting that synthetic curare-like drugs ought to be tested on a variety of species.

S. L. W.

PHARMACY DISPENSING

Fatty Oils, Neutralisation of, for Injection. C. G. van Arkel and J. J. M. van Sonsbeek. (*Pharm. Weekbl.*, 1949, 84, 70.) Neutralisation of fatty oils is sometimes carried out by shaking the oil with excess of calcium or magnesium oxide, possibly with the addition of a trace of alcohol. The method was found effective in reducing the natural acid value of an oil from 0.32 to 0.16 (with magnesia) or 0.09 (with lime). Traces of these metals could be detected in the neutralised oils. When using an oil to which fatty acid had been added to give an acid value of 9.63, it was found necessary to add water in order to obtain a satisfactory result, but too much water causes formation of emulsions and difficulty in filtration. The dissolved metal amounted to, for magnesium 0.2 mg./100 ml., and for calcium, 6.5 mg./100 ml. If, however, the filtration is carried out with the aid of heat, larger quantities are dissolved. It is concluded that the method of neutralisation with soda is to be preferred on account of greater reliability and the possibility of filtration at a raised temperature.

G. M.

Penicillin Ointment, Stability of. S. H. Culter. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 370.) A number of penicillin ointments, using various types of bases, with sodium, potassium, and calcium salts of varying degrees of potency were prepared. These ointments were stored in collapsible tin tubes at room and refrigerator temperatures and assayed from time to time to determine the stability of the penicillin. It was found that impure sodium penicillin (400 I.U./mg.) is very unstable in an aqueous or a non-aqueous water-miscible ointment base and has only limited stability in an anhydrous petrolatum base. Calcium penicillin (500 to 650 I.U./mg.) is much more stable than the impure sodium salt in the same bases, while the high potency (1583 to 1620 I.U./mg.) crystalline sodium or potassium salts are equal, if

N-phenyl anthranilic acid was studied in detail and adopted for the estimation of urea in blood. Investigations of the reaction conditions were made involving a study of the quantities of reagent used, the time of heating, the development of the colour and the specificity of the reaction. Calibration and absorption curves are given for the reaction colour, which obeyed Beer's law up to a urea concentration of 20 $\mu\text{g.}/\text{ml.}$ Under the conditions prescribed ammonia, histidine, tyrosine, cystine, caffeine, uric acid, barbiturates, acetamide, asparagine, creatine, sulphonamides and thiouracil all give negative reactions. Proteins and monosubstituted ureas give red colorations; semicarbazide gives a magenta colour similar to that obtained with urea, while biuret gives a brown colour. Creatinine gives a positive reaction, but fortunately the sensitivity in this case is only one hundredth of that with urea, so that creatinine will not interfere with the estimation of urea in biological fluids. The method showed fairly good agreement with the manometric hypobromite method except that in 10 per cent. of cases errors of 8 to 10 $\text{mg.}/100 \text{ ml.}$ were obtained. The estimation can be performed with as little as 0.2 ml. of blood and compares favourably with other colorimetric urea determinations, although it is not sufficiently accurate for urea clearance tests.

R. E. S.

CHEMOTHERAPY

Fungistatic Activity and Structure in a Series of Simple Aromatic Compounds. G. W. K. Cavill, J. N. Phillips and J. M. Vincent. (*J. Soc. chem. Ind., Lond.*, 1949, 68, 12.) Derivatives of benzene are assessed for activity against *Aspergillus niger*. For comparison, the logarithm of the reciprocal of the millimolar concentration giving 50 per cent. inhibition is used to express fungistatic activity. In the case of ionised substances, this is calculated relative to the un-ionised form as there is some evidence that the ionised forms are not so active fungistatically. Benzene and toluene have a small activity, aniline, benzoic acid, phenol and nitrobenzene are less active, but chlorobenzene is more active. Saturated compounds are considerably less active than the corresponding aromatic substances. In general, halogen substitution increases activity. Substitution of aniline, benzoic acid or phenol with hydroxyl or amino groups nearly always reduces activity, except that -OH substituted *ortho* to carboxyl groups increases activity slightly. *Meta* or *para* substitution of a carboxyl group in phenol or in aniline decreases activity, but *ortho* carboxyl groups cause an increase, or little change, in activity. Nitration of phenol or aniline increases activity (except for picric acid and trinitroresorcinol which are ionised), but nitrobenzoic acid is less active than benzoic acid. The introduction of $-\text{CH}_2-$ groups between ring and carboxyl group does not enhance the activity of benzoic acid, and decreases that of 4-aminobenzoic acid; the same applies to the methyl esters. To conform to the conclusions above, 4-hydroxybenzoic acids have to be regarded as substituted benzoic acids, not as substituted phenols. Alkylation, alkyl esterification or alkyl etherification generally increases activity. There is some correlation between reciprocal water solubility and activity for a homologous series, but this breaks down if a wider range of compounds is taken. Slightly better agreement is obtained when the logarithm of relative solubility in alcohol and water is compared with activity, but there are considerable deviations.

G. B.

Polymethylene bis-Quaternary Ammonium Salts, Curare-like action of. R. B. Barlow and H. R. Ing. (*Brit. J. Pharmacol.*, 1948, 3, 298.) The following series of polymethylene bis-quaternary ammonium dibromides

malarial activity has been established. It is an evergreen shrub growing in Szechuan and Yunnan; the dried roots are known as *Ch'ang Shan*. In Yunnan the leaves and twigs are used and these have been shown to be much more active than the roots.

J. W. F.

Belladonna, Indian, Pharmacognosy of. H. W. Youngken and W. E. Hassan, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 450.) A complete description and comparison of this plant with *Atropa Belladonna* Linn. is given. The materials used were grown by the workers from authenticated seeds and surplus material has been deposited in the Herbarium of the Massachusetts College of Pharmacy. *Leaves*: the following data are reported; *Vein Islet Number*, species average = 10; *Palisade Ratio*, 5.0 - 8.3 - 12.4 (*A. Belladonna*, 4.5 - 6.9 - 9.2), *Stomatal Index*, Upper Surface, 3.4; Lower surface, 17.6; (*A. Belladonna*, 2.9 and 17.6 respectively). *Floral Members*; the Indian variety has larger flowers than European, and the corolla is bright yellow. *Roots and stems*: similar basic structure except that the cells of the Indian variety are larger than those of the European. *Alkaloidal content*: the Indian variety has a high alkaloidal content. Drying under infra-red lamps at 145° C. destroys the alkaloidal content. No significant increase in alkaloidal content resulted from the injection of amino-acids into the growing plant. The authors suggest that Indian belladonna is a variety of the European and should be named *Atropa Belladonna* variety *acuminata*.

J. W. F.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and norAdrenaline, Action on Human Heart-rate. H. Barcroft and H. Konzett. (*Lancet*, 1949, 256, 147.) The actions of intravenous infusions of noradrenaline and of adrenaline on the heart-rate and arterial blood pressure of normal men and women have been studied. In doses of 10 to 20 µg./minute noradrenaline causes bradycardia, whereas adrenaline causes tachycardia. Subjective effects during the adrenaline infusions included mild palpitation, hyperventilation, tightness in the chest, and muscular fatigue; there were usually no subjective symptoms during infusions of noradrenaline. The explanation of the different actions of adrenaline and noradrenaline on the heart-rate is not known, but the authors suggest that two factors are probably concerned—the direct excitatory action of the drug on the pacemaker, and its reflex inhibitory action due to its pressor action on the vascular system.

S. L. W.

***Courbonia virgata*. Identification of Toxic Principle as a Tetramethylammonium Salt.** A. J. Henry. (*Brit. J. Pharmacol.*, 1948, 3, 187.) The tuberous root of *Courbonia virgata* A. Brongn. (Fam. Capparidaceae), a plant occurring in the Southern Sudan, Northern Uganda, Kenya, and French Equatorial Africa, has been found to contain a toxic principle, tetramethylammonium iodide. This was also found in the scaly shoots and the superstructure, and in the leaves of the subsidiary shoots. It has been named tetramine and the fresh root contains about 0.2 per cent. About 0.25 g. of the base taken orally (in the form of the root) has proved lethal to adult human beings within an hour. Lethal dose of the iodide subcutaneously was 0.5 to 1 mg./25 mg. of mouse, the symptoms being convulsive spasms, collapse, and death within 30 minutes. Intravenous injection of 8 mg. of the iodide into a rabbit caused death within 2 minutes. The

ABSTRACTS

not superior, to the impure calcium salt for ointment purposes in respect of stability. Penicillin ointments are somewhat more stable at refrigerator temperatures than at higher temperatures. The inclusion of sulphadiazine, sulphathiazole, adrenaliné, or benzocaine in a penicillin ointment does not materially influence the stability of the penicillin, but water, zinc stearate, aluminium hydroxide gel hasten its deterioration to a marked degree.

S. L. W.

Penicillin Powders, Preparation of. J. Büchi and F. O. Gundersen. (*Pharm. Acta Helvet.*, 1949, 24, 31.) A penicillin powder, prepared by dilution of penicillin with lactose, had a limited stability, since 15 per cent. of the activity was found to be lost after keeping for 2 months at 4°C. Preparations made according to the following formula were more satisfactory and showed no loss after 4 months at 4°C., provided that the materials were dried thoroughly before use, and the powder was kept over calcium chloride. Crystalline sodium penicillin, 1,000,000 units; hydrogenated arachis oil (m.pt. 37°C.), 0.25 g.; anæsthetic ether, 5 ml.; sodium laurylsulphate, 0.50 g.; de-enzymated gum acacia, 1.50 g.; diluent, to 50.00 g. In the preparation the penicillin is rubbed down with a solution of the hardened arachis oil in the ether, in order to coat the particles of penicillin, and the other substances are then added. The mixture is finally passed through sieve VI (approx. 100 mesh/inch; wire 0.08 mm. diam.). Anhydrous lactose, sulphanilamide or dried milk may be used as diluent.

G. M.

Sterility of Chemicals, Employment of Filtration in Testing. O. Bang, G. Bowitz and A. T. Dalsgaard. (*Arch. Pharm. Chem.*, 1949, 56, 643.) The authors have examined the method of testing for sterility proposed by Davies and Fishburn (*Quart. J. Pharm. Pharmacol.*, 1946, 19, 36). Their results show that the risk of infection arising during manipulation cannot be ignored, since out of 113 tests (30 with a dry filter, the others with sterile solutions) 15 gave positive results. Tests were carried out with a number of pure chemicals, and positive results were obtained with a proportion in the cases of ascorbic acid, hexobarbitone, morphine hydrochloride, dextrose, allylisopropyl barbitone, phenobarbitone, oxedrine tartrate, benzocaine, and boric acid. "Sterilised" boric acid powder gave positive results in 9 tests out of 10. Generally the contaminating organism was a Gram-positive rod. The authors consider that the method is worthy of further study and possible official adoption.

G. M.

PHARMACOGNOSY

Antimalarial Plants, Chinese. S. T. Yang. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 458.) A brief description of five Chinese medicinal plants which may have antimalarial activity is given. 1. *Fraxinus malacophylla* Hensl. This tree occurs in S.E. Yunnan and is known as *Pei Chiang Kan*; the root-bark is used. Recent tests indicate the absence of antimalarial activity. 2. *Fraxinus chinensis* Roxb. Grows in Szechuan and Yunnan; the bark yields fraxetin which earlier workers claimed to possess antimalarial activity; however recent work indicates it is ineffective. 3. *Clerodendron yunansis* Hu. Grows near Kunming where thin slices of the twigs of this tree are sold as *Tien Ch'ang Shan*. No investigations have been conducted on this plant. 4. *Alstonia yunansis* Diel. Also grows near Kunming; the bark, twigs and pods are sold as *Chih Ku Ch'ang Shan*. Several alkaloids and a resinous substance are present, but they show no antimalarial activity. 5. *Dichroa febrifuga* Lour. This is the only plant in the group whose anti-

were found to depress the isolated heart and dilate the coronary vessels. The activity of these drugs as local anaesthetics on the frog's lumbar plexus appeared to be more nearly related to their activity against acetylcholine than to their activity against histamine; benadryl and 3277 RP were, in fact, much more potent than antistim and neoantergan both as local anaesthetics and as acetylcholine antagonists.

S. L. W.

Isomeric Heptylamines, Comparative Pharmacology of. D. F. Marsh, (*J. Pharmacol.*, 1948, 94, 225). In this study the vasopressor activity of the isomeric heptylamines in anaesthetised and unanaesthetised dogs was determined and compared with adrenaline. By limiting the investigation primarily to compounds with a total of seven carbon atoms it was possible to determine the relationship between spatial configuration and pharmacological activity without having to consider differences in molecular weight. The most potent of the compounds was 4-methyl-2-heptylamine which is about 1/200 as active as adrenaline and has a long duration of action; orally, the heptylamines have but little pressor action in man. With the exception of the 4-heptylamine, they increased the tone of isolated rabbit jejunum with a concentration of 4 mg./100 ml., caused contraction of the rat uterus and antagonised the relaxant action of adrenaline. In the perfused heart they produced a decrease in rate, force of contraction and outflow of perfusate. They do not antagonise histamine constriction in the perfused guinea-pig lung.

S. L. W.

Isuprel in Spontaneous and Induced Asthma. F. C. Lowell, J. J. Currey and I. W. Schiller. (*New Engl. J. Med.*, 1949, 240, 45.) Isuprel, 1-(3: 4-Dihydroxyphenyl)-2-isopropylaminoethanol, (isopropyladrenaline) has been studied for a number of years in Europe under the name of aleudrin, and has been advocated for use in the form of an aerosol for the relief of attacks of bronchial asthma. The authors report their observations of the administration of isuprel to asthmatic subjects in the out-patient clinic, in the wards, and in the laboratory (induced asthma). In most cases the drug was given as an aerosol, in a concentration of 1.0 and 0.5 per cent., but it was subsequently given in tablets sublingually containing 10 mg., or subcutaneously or intramuscularly in a concentration of 0.02 or 0.01 per cent. Given by aerosol it was very effective in relieving mild or moderately severe asthma and appears the most effective agent available for self-medication; in severe and prolonged attacks it was far less satisfactory. In certain cases other medication, particularly aminophylline intravenously, was required; on recovery, isuprel was again effective in the control of milder attacks. Sublingual and parenteral administration of the drug was not very effective. Side-effects were uncommon in the doses used in this study.

S. L. W.

Procaine, Influence of Potassium and Calcium Ions on. H. J. Bein. (*Brit. J. Pharmacol.*, 1948, 3, 251.) The action of procaine on the refractory period of the isolated rabbit auricle was determined in the presence of varying amounts of potassium and calcium. The influence of potassium and calcium was shown to be antagonistic; increasing the amount of potassium or decreasing the amount of calcium both potentiated the action of procaine, about the same potential change being obtained by raising the K by 50 per cent. or by lowering the Ca by 50 per cent. A reduction of the potassium or an increase of the calcium content produced the same effect qualitatively but not quantitatively. The action of procaine was depressed, but to obtain

toxic principle is not extracted from alkaline solution by organic solvents and unless its presence is suspected and its properties known, it might easily be overlooked. The toxic properties of the plant are well known to the natives. As a qualitative test for tetramine, the crystalline precipitate which it produces with Wagner's reagent can be used. From warm dilute solutions, either acid or neutral, the periodide rapidly separates as well-shaped rhombic crystals which are readily recognised under the microscope. S. L. W.

Dextran as a Plasma Substitute. J. J. Bull, C. Ricketts, J. R. Squire, W. d'A. Maycock, S. J. L. Spooner, P. L. Mollison, and J. S. C. Paterson. (*Lancet*, 1949, 256, 134.) Dextran is produced by the growth in culture of certain micro-organisms, in particular of *Leuconostoc mesenteroides*, in a substrate of glucose and phosphate. After removal of protein and inorganic salts from the culture fluid dextran is precipitated as a syrupy gum by organic solvents such as acetone; so obtained, it is a polysaccharide composed entirely of glucose units. The molecules of this crude dextran are too large for infusion purposes and preparations of smaller molecular size are produced by partial hydrolysis with acid. In defining a specification of dextran for intravenous use the proportion of dextran of low molecular weight should be kept to a minimum; it is probably also important to define the upper limit of molecular size. Physico-chemical methods for controlling molecular size are described. The solution for infusion is colourless or pale straw colour and of about the same specific gravity and saline concentration as plasma, with a colloid osmotic pressure 1.5 to 2.0 times that of normal plasma. It is well tolerated as an infusion by man and is not pyrogenic, toxic, or antigenic. Immediately after infusion the erythrocyte-sedimentation rate is increased and rouleaux can be observed in smears of blood. No increase in the osmotic fragility of the red cells has been observed. Dextran has proved efficacious as a plasma substitute in cases of burns and has produced a sustained increase in the venous return in patients with surgical shock or hæmorrhage, but as there is still doubt as to the ultimate fate of dextran in the body it cannot yet be recommended unreservedly for intravenous infusion. S. L. W.

Histamine Antagonists, Comparison of. J. J. Reuse. (*Brit. J. Pharmacol.*, 1948, 3, 174.) Comparison of a number of histamine antagonists on isolated organs placed them in the following order of descending activity: neoantergan, 3277 RP, benadryl, antistin, nupercaine. Besides being the most active of these drugs against histamine, neoantergan is also the most specific; it had the least action against acetylcholine, and its action against nicotine, potassium and adrenaline was much smaller than its action against histamine. It is thus clearly the best of the drugs studied to use in specific tests for histamine in unknown solutions, but it is useless if high concentrations are used. A satisfactory method for carrying out tests of this kind is first to find doses of the tissue extract and of histamine which cause equal effects on a piece of guinea-pig's ileum, and then to continue giving these doses alternately and to study the effect, in a series of responses, of a brief addition (1 minute) of a small dose of neoantergan to the bath. The dose of neoantergan is chosen so as to produce 50 to 70 per cent. inhibition of the subsequent response to histamine. The concentration of neoantergan for this effect is usually about 1/10 the concentration of histamine. A method for the rough biological assay of neoantergan is described which involves the use of only about 0.002 μg . of the drug per dose. The antihistamine drugs

effect of these pyrogenic materials, indicating that the pyrogen produced by the organisms is not a significant factor in the production of fever. It was, however, found that tolerance developed on repeated injection of pyrogenic material during fever, showing again that the pyrogen produced by *E. coli* is not the main factor in the causation of a raised temperature. It is suggested that perhaps a product of cell injury is the cause of the fever. Similar experiments were carried out with the injection of sterile exudates of acute inflammation, the exudates being produced by the intrapleural injection of turpentine in dogs. Daily injection of exudate produced no tolerance to its fever-producing effect. Animals tolerant to pyrogens remained fully responsive to exudate. The fever-producing property of exudates is not therefore due to the presence of bacterial pyrogen.

H. T. B.

β -Pyrrolidine-ethyl-phenothiazine (Pyrrolazote), Pharmacology of. M. J. Vander Brook, K. J. Olson, M. T. Richmond and M. H. Kuizenga. (*J. Pharmacol.*, 1948, 94, 197). This compound was compared with pyribenzamine and was shown to be highly specific as a histamine antagonist. It appears to be effective for a longer period of time than pyribenzamine as judged by both the activity it exhibits against histamine spasms of smooth muscle *in vitro* and the protection it affords against fatal histamine intoxication by aerosol *in vivo*. It possesses anti-anaphylactic properties similar to those of pyribenzamine. Pyrrolazote has no effect on the pressor responses to adrenaline; in this respect it differs from pyribenzamine, benadryl and neoantergan, all of which enhance the pressor response. Acute toxicity experiments show that pyrrolazote is considerably less toxic than pyribenzamine in mice, rats and rabbits, and chronic toxicity studies in rats showed that a dose of 10 mg./kg. orally 5 days each week for 10 weeks produced no gross pathology, and growth was not impaired. Histopathology limited to degenerative fatty infiltration of the liver occurred at doses of 25 mg./kg. and greater.

S. L. W.

Quinine Methiodide, Pharmacology of. F. H. Shaw, P. Keogh and M. MacCallum. (*Austral. J. exp. Biol.*, 1948, 26, 147). The authors show that while quinine methiodide retains many of the properties of quinine it has also a curare action on the neuromuscular junction and sympathetic ganglia. It weakens the depressor action of adrenaline and in this respect is the complement of yohimbine and ergotoxine. Because of its extreme toxicity towards the respiratory centre it would be an unsuitable clinical substitute for curare. Intravenous doses of as low as 10 mg./kg. in the cat or dog nearly always resulted in immediate cessation of respiration. It is suggested that it may provide another useful pharmacological test for adrenaline.

S. L. W.

Thyroid Activity, Biological Assay of. D. E. Hutcheon (*J. Pharmacol.*, 1948, 94, 308). The results of two relatively simple methods for estimating the physiological potency of thyroxine are presented. One is a quantal response type of assay depending on the decreased resistance to anoxia of mice treated with thyroxine. For this, adult mice, weighing from 20 to 25 g., were divided into 4 groups of 10 animals, one group serving as a control while the other 3 were given thyroxine 2.5, 5.0 and 10.0 μ g. subcutaneously daily for 7 days. 48 hours after the last injection the mice were all placed in an air-tight chamber of 32 litres capacity containing soda-lime. When approximately half the mice had died the survivors were removed and the mortality-rate of each group noted. The method of calculating the

(Continued on page 421)

the same degree of depression as that obtained by reducing the K concentration by 50 per cent. it was necessary to increase the Ca concentration by 600 per cent. The determining factor is therefore not the ratio K/Ca but the absolute amount of potassium present, though the presence of at least the normal amount of Ca is necessary.

S. L. W.

Proguanil (Paludrine), Intravenous. R. N. Chaudhuri and H. Chakravarti. (*Brit. med. J.*, 1949, 1, 91.) Proguanil acetate was administered by intravenous injection to 11 patients ranging in age from 9 to 60 years, in 8 of whom *Plasmodium falciparum* infection was predominant, while 2 had *P. vivax* infection and 1, mixed infection. Four patients were gravely ill with pernicious symptoms, 4 had heavy parasitic infection with frequent vomiting, and 3 had "ordinary" malaria. Doses varied from 25 to 400 mg., and were repeated in a few cases, the total amount injected ranging from 200 to 600 mg. Although the series was too small to determine the best dosage, in the majority of cases 200 to 400 mg. produced a striking effect, controlling the temperature and clearing the peripheral blood of asexual parasites in 2 or 3 days. One patient admitted in a moribund state, died; another, with typical cerebral malaria, remained unconscious for several days and later developed signs and symptoms of encephalitis, from which, however, he recovered completely. The injections were well tolerated, although 2 patients complained of pain along the injected vein, possibly due to some local phlebitis. A footnote to this paper reports that proguanil lactate is more soluble and less irritant than the acetate, and is being used by intramuscular injection.

G. R. K.

Proguanil (Paludrine) in Prophylaxis and Treatment of Malarial Infections caused by a West African Strain of *Plasmodium falciparum*. G. Covell, W. D. Nicol, P. G. Shute and M. Maryon. (*Brit. med. J.*, 1949, 1, 88.) Proguanil was found to act as a true causal prophylactic of infections of the strain of *Plasmodium falciparum* used, and the prophylactic dose recommended for non-immune adults exposed to malarial infection in West Africa is 100 mg. daily. Although it controlled the clinical attack caused by infections with the same strain, its action in this respect and in clearing asexual parasites from the peripheral blood was somewhat less rapid than that of mepacrine or quinine, and by itself, it did not effect a radical cure. Nevertheless, a course of 300 mg. twice daily for 10 days effectively sterilised the gametocytes and rendered them non-infective to mosquitos for as long as they continued to be present in the peripheral blood in sufficient numbers for infection to occur. Proguanil also has the lowest toxicity of any known antimalarial drug. In the treatment of *P. falciparum* malaria infections, rapid termination of the clinical attack, a high radical cure rate, sterilisation of the gametocytes and minimum risk of injurious side-effects is achieved by a course of 300 mg. of proguanil twice daily for 10 days with 900 mg. of mepacrine given in 3 doses on the first day, followed by a maintenance dose of 100 mg. of proguanil for the ensuing 6 weeks.

G. R. K.

Pyrogens and Fever of Acute Infection. J. L. Bennett. (*J. exp. Med.*, 1948, 88, 267, 279.) The possibility that the fever accompanying acute infections is a response to a pyrogen produced by the infecting organism was investigated in rabbits. Animals surviving dermal pneumococcal infections, or peritonitis due to *Escherichia coli*, were given intravenous injections of typhoid or *E. coli* vaccine. They showed no tolerance to the fever-promoting

LETTERS TO THE EDITOR

The Assay of Mersalyl

SIR.—Methods of assay of mersalyl [sodium salt of salicyl-(2-hydroxymercuri-2-methoxypropyl)amide-O-acetic acid], phenylmercuric nitrate, and *ciguatum hydrargyri nitratis dilutum* as proposed by Waterhouse¹ and modified by Pierce² have become official in the British Pharmacopoeia, 1948. All involve reduction to metallic mercury under reflux, solution of the precipitated mercury in nitric acid, and titration with ammonium thiocyanate. It was found that erratic results were sometimes obtained in the assay of mersalyl, and also when this assay process was applied to injection of mersalyl. An investigation was therefore undertaken into the cause. Under the conditions of assay, mercury being to some extent volatile, it was found that vigorous boiling caused condensation of the metal on the cold part of the reflux condenser, mercury in such a form often being difficult to wash off. It is suggested, therefore, that glass jointed apparatus be used, and that after the reduction, the condenser should be washed with water and the precipitate transferred to the filter paper as directed. Nitric acid (20 ml.) and water (10 ml.) are then placed in the flask and refluxed in the apparatus for 10 minutes. The condenser is finally washed with 10 ml. of water, and the acid, after cooling, used to dissolve the zinc amalgam in the usual way, the assay then being completed in accordance with the directions given.

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The British Drug Houses, Ltd.,
City Road, London, N.1.
March 30, 1949.

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1. Waterhouse, *Quart. J. Pharm. Pharmacol.*, 1938, 11, 458.
2. Pierce, *ibid.*, 1942, 15, 367.

ABSTRACTS (continued from page 419)

slope of the dose-mortality curve was that described by Finney, allowances being made for the mortality rate of the control groups. The weighted mean slope was found to be 3.53 with a standard error of ± 0.76 . The other method is a graded response method based on the acute weight loss of rats following thyroxine injections. For this, adult male albino rats, weighing 220 to 280 g., were given drinking water containing 0.1 per cent. of thiouracil for 10 days prior to injection. They were then placed in individual cages and assigned at random to the various treatments; food and water were not restricted. Two subcutaneous injections of 0.25 to 2.0 mg. of thyroxine were given on successive days. The body weights were measured for several days before and after the injections. The data obtained from two experiments are presented and were subjected to an analysis of variance according to the method of Bliss and Marks (*Quart. J. Pharm. Pharmacol.*, 1939, 12, 82, 182). The mean deviation of this method was 4.4 and the slope of the log dose response curve was 9.1; the value for λ was therefore 0.478. Though this method is easily performed in a very short time, a very large number of animals is necessary (400). The asphyxiation test is approximately as accurate and its precision is of the same order as that of other assays based on the all-or-none response.

S. L. W.

BOOK REVIEWS

THE PLANT ALKALOIDS, by T. A. Henry. 4th edition, 1949, p.p. xxii + 783 and Index. J. and A. Churchill, Ltd., London.

The international reputation of Henry's "Plant Alkaloids" is such that workers in many branches of science throughout the world will be grateful that Dr. Henry has been able to keep track of, digest, and present in his usual lucid and authoritative manner, the wealth of information that has been published on topics relating to the plant alkaloids and their analogues in the past decade since the third edition appeared. A large part of the volume has had to be rewritten and its bulk continues to increase. Not only have the years since 1939 brought to light a considerable volume of new knowledge of the occurrence and distribution of alkaloids in plants, of their biogenesis, isolation, purification and properties, chemical structure and pharmacological action, but they have also led to very extensive work on the preparation and study of synthetic analogues. Perusal of the edition under review has failed to reveal any important or less important publication having a bearing on plant alkaloids that has not received Dr. Henry's attention. As in the previous edition the material is classified primarily on the basis of nuclear structure although it is admitted that the basis adopted must necessarily be arbitrary in many cases where chemical complexity is such that the structures could have been accommodated under more than one nuclear heading. Two new groups have been required to accommodate new types encountered, namely the pyrrolizidine group comprising so far principally the necine derivatives found in *Senecio* species and the steroidal alkaloid group which includes the alkaloids of *Aconitum*, *Delphinium* and *Veratrum* species and the glucosidal alkaloids of *Solanum* species. The difficulty of keeping pace with additions to knowledge in this field is shown *inter alia* by the fact that while the text was going through the press evidence was published of the steroidal nature of the alkaloid conessine, derived from Kurchi bark which has recently attracted much attention from pharmacologists. A reference to the recent assignment of an *allopregnane* structure to conessine has been inserted in the introduction, though the *Holarrhena* alkaloids had necessarily to be dealt with in the body of the work under "Alkaloids of Undetermined Constitution." Alkaloids derived from an acridine nucleus have recently been isolated from certain members of the *Rutaceae* in Australia and, as Dr. Henry points out, a new group will require to be added to his classification to accommodate these. The literature on synthetic analogues of alkaloids is duly referred to and the repercussions of the extensive war-time work on synthetic potential antimalarials on the correlation of structure with action are taken into account. Due attention is paid to the biogenesis of alkaloids, including Robinson's recent discussions on strychnine and emetine.

In addition to the physical, physicochemical and chemical properties of something like two thousand alkaloids and their derivatives, references are included to the newer techniques applied to alkaloidal analyses, including micro-methods of detection and estimation and the application of procedures involving chromatographic and polarographic methods. All who are concerned, however remotely, with the plant alkaloids and related chemistry will require to ensure that this new edition, which is as carefully written and produced as its predecessors, is among their available works of reference.

F. HARTLEY.

LETTERS TO THE EDITOR

The Assay of Mersalyl

SIR.—Methods of assay of mersalyl [sodium salt of salicyl-(γ)-hydroxymercuri- β -methoxypropyl]amide-O-acetic acid], phenylmercuric nitrate, and unguentum hydrargyri nitratis dilutum as proposed by Waterhouse¹ and modified by Pierce² have become official in the British Pharmacopœia, 1948. All involve reduction to metallic mercury under reflux, solution of the precipitated mercury in nitric acid, and titration with ammonium thiocyanate. It was found that erratic results were sometimes obtained in the assay of mersalyl, and also when this assay process was applied to injection of mersalyl. An investigation was therefore undertaken into the cause. Under the conditions of assay, mercury being to some extent volatile, it was found that vigorous boiling caused condensation of the metal on the cold part of the reflux condenser, mercury in such a form often being difficult to wash off. It is suggested, therefore, that glass jointed apparatus be used, and that after the reduction, the condenser should be washed with water and the precipitate transferred to the filter paper as directed. Nitric acid (20 ml.) and water (10 ml.) are then placed in the flask and refluxed in the apparatus for 10 minutes. The condenser is finally washed with 10 ml. of water, and the acid, after cooling, used to dissolve the zinc amalgam in the usual way, the assay then being completed in accordance with the directions given.

P. S. STROSS.

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March 30, 1949.

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1. Waterhouse, *Quart. J. Pharm. Pharmacol.*, 1938, 11, 458.
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F. HARTLEY.

not only stable towards cholinesterase, but inhibit the action of cholinesterase on acetylcholine in a reversible manner. A similar action had been described for *bis-p*-dimethylaminobenzylacetone dimethiodide and for various alkyl phosphates, although the latter combine irreversibly with cholinesterase. The "stigmines" act against the effect of curare, but are seemingly not able to counteract the curariform action of *bis*-trimethylammonium decane.

While the nicotinic action of acetylcholine is antagonised either by ganglia-blocking agents, such as tetraethylammonium salts or *bis*-trimethylammonium pentane, or on the skeletal muscle side by curariform compounds, the muscarinic action of the cholinergic neuro-hormone and parasympathomimetics is antagonised by atropine. This drug, together with similarly acting compounds, named parasympatholytics, and the papaverine and anti-histamine group, form what one may call the larger group of anti-spasmodics or spasmolytics. There is hardly any representative of this group which does not possess a multiplicity of action but usually one predominates over the others, either the atropine-like, the musculotropic papaverine-like, or the histaminolytic. The parasympatholytics or anti-acetylcholine drugs represent mostly nitrogen-containing esters which, when Pfeiffer's theory of prosthetic distances is applied, show similar distances between the nitrogen-methyl group and the two oxygens to those of the protagonist group, the parasympathomimetics. This represents a very neat illustration of the receptor theory.

On looking through the list of drugs of the parasympatholytic group, we find that the acid part of the ester is "heavier" than acetic acid, Pfeiffer's umbrella effect being obtained by additional phenyl or hydroxy groups. The alkanolamine part need not be so complicated as the tropine of atropine, but can be represented by dialkylamino alkanols. The original tropic acid has been changed in time to mandelic, benzilic, diphenylacetic and fluorene carboxylic and dihydroanthracene carboxylic acids. Compounds prepared for different purposes, but showing, superficially, similarities with the atropine group, except for the fact that they are not esters, show varied amounts of parasympatholytic activities; but even compounds like Benadryl or diphenylpropylamines, without oxygen, still possess measurable anti-acetylcholine action in addition to anti-histamine and anti-barium activities.

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SCIENTIFIC MEETINGS

SOME ASPECTS OF PHARMACOLOGICAL CHEMISTRY

By F. BERGEL, D.Sc., F.R.I.C.

A Summary of Three University Lectures given in the Department of Pharmacology, University College, London. January-February, 1949.

ONLY the symptomatic drugs were considered in this series. The first lecture dealt with the significance of total chemical constitution and the theories of modes of drug action¹. Examples indicating constitution-action-parallelism were quoted, but it was emphasised that much more data were required to produce a more secure basis for a rational approach. In the next lecture the work on the analgesic substances, particularly on morphine, was discussed². On the third occasion the chemistry of the parasympathomimetics and parasympatholytics was considered. These drugs are based on acetylcholine, which is not strange to the organism. Acetylcholine has three pharmacological actions, muscarinic, nicotinic and under certain conditions, curare-like. As an ester, it is hydrolysed by the enzymes, cholinesterases of blood cells and plasma and transformed into the weakly active choline. Its re-synthesis may be due to the action of an acetylase, the co-enzyme of which may consist partly of pantothenic acid. Its existence was demonstrated by Feldberg and Mann in brain and by Bülbring and Burn in rabbit heart auricles. There exists also an inactive acetylcholine complex, the formation of which may partly explain the disappearance of the neurohormone. That it also occurs in plants was shown by Feldberg's discovery of acetylcholine in the stinging nettle. The instability and amphotropic properties of acetylcholine stimulated the pharmacological chemist to synthesise substances with more sustained and clear-cut action. When considering substances which, like acetylcholine, possess parasympathomimetic action, Pfeiffer's theory of prosthetic³ distances comes to mind, postulating an optimum distance between the N-methyl group and the two oxygen atoms. Thus it can be understood why three natural drugs—muscarine, pilocarpine, and arecoline—show, on the whole, lower activities and have never gained clinical importance. Virtual changes of the acetylcholine molecule itself have produced evidence that the free aminoalkanols are very much weaker and that, for the existence of full muscarinic action, the alcohol group must be esterified and the nitrogen carry at least two methyl groups. When the chain of the alcohol or the acid is elongated, activity falls considerably. There is one exception, and that is when the aminoalkanol chain is branched, as in mecholyl which shows strong muscarinic effect. Acetic acid has been exchanged against other acidic residues, such as carbamic acid in carbachol, thioacetic acid, etc. Transformation of the choline ester into choline ethers produces more stable but less active compounds. An ether-like product was made by Fourneau (2268 F) and found to be very potent indeed. Another ether-like compound is Esmodil which contains a double bond like arecoline and croton betaine methyl ester. The latter, carrying the nitrogen on the acidic side, is very much like acetylcholine though weaker. While choline itself shows very little activity, 3-hydroxymethylpyridine, the alcohol corresponding to nicotinic acid, possesses interesting parasympathomimetic properties. Other aromatic compounds with phenolic groups in place of alcoholic hydroxy groups, show remarkable properties, especially when esterified with carbamic acid. Such substances, like physostigmine, Prostigmine (neostigmine) are

REVIEW ARTICLE

MATERIALS USED IN GREAT BRITAIN FOR THE ACTIVE IMMUNISATION OF MAN AGAINST DIPHTHERIA

BY SIR PERCIVAL HARTLEY,
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FOLLOWING the introduction of antitoxin for the treatment of diphtheria in 1895 there was a fall in the case-fatality in these islands which, some ten years later, had reached about one-third of that of pre-antitoxin days. Although the rate declined a little further, and fluctuated from year to year, it persisted at such a level that diphtheria remained as a major concern for public health officers and the authorities of infectious diseases hospitals, calling for a sustained and intensive effort in the field by the former and the maintenance of an expensive hospital service to provide for the latter. In spite of all the measures taken, however, in the ten years prior to the outbreak of the second world war, there were from 2,500 to 3,000 deaths from diphtheria every year in this country, and this rate continued until 1941. In 1947, however, the deaths had reached the low record—up to then—of 244 for that year, and the (uncorrected) figure for 1948 is even lower at 156. Since a decline in case-fatality rate may be independent of measures taken for prevention or treatment, it may be prudent to reserve a final judgment to a more detailed examination of all the factors concerned; but the evidence at present available is that active immunisation has contributed very materially to the extremely low incidence and mortality from diphtheria in recent years, and many take the view that this has been the dominating factor.

The materials which have been used, and have thus made their contribution to this outstanding achievement of public health endeavour, have attracted the continued interest of research workers in English laboratories from the early 1920's and the preparations used, in this country exclusively and in other countries to a considerable extent, are largely the fruits of their labours. Accordingly, it may be of interest to those whose duty it is to supply these materials, to keep them before the notice of our people and advocate their continued employment in the future, to know something of their historical development, their nature, preparation, composition, properties and use.

Although the value of antitoxin for prophylaxis and therapy was recognised, it was obvious that protection from attack was of greater importance than the treatment of individual cases and the passive immunisation of contacts: so long as susceptible populations remained

NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Atophan* balsam contains amyl ester of phenylcinchoninic acid 10 per cent., phenyl salicylate 10 per cent., camphor $2\frac{1}{2}$ per cent., and synthetic menthol $2\frac{1}{2}$ per cent., incorporated in a neutral base. It is indicated in all forms of acute and chronic muscular rheumatism, especially lumbago, and in arthralgia resulting from rheumatism and gout, where local medication and massage are required simultaneously. It is supplied in jars containing $2\frac{1}{4}$ ounces, and in tubes of 50 g.

S. L. W.

Atophanyl* is a solution of equal parts of atophan sodium and sodium salicylate in sterile distilled water for parenteral administration in rheumatic and gouty affections of the muscles and joints. Injections are given either intravenously or intramuscularly, the contents of one ampoule being injected daily until the symptoms have disappeared, with an interval of 2 or 3 days after each 4 consecutive injections. The ampoules for intravenous injection contain $7\frac{1}{2}$ grains of each substance in 10 ml.; for intramuscular injection the same doses are contained in 5 ml. It is intended for the relief of severe acute symptoms and not for prolonged treatment; a course of more than 15 injections is rarely called for. Atophanyl is issued in boxes of 5 ampoules of 10 ml. for intravenous injection, and boxes of 5 ampoules of 5 ml. for intramuscular injection (the intramuscular ampoule contains $4\frac{1}{2}$ grains of urethane as a local anæsthetic).

S. L. W.

Methergin*, methylergometrine tartrate, is *d*-lysergic acid-*n*-butanol-diamide, and differs structurally from ergometrine in possessing a butanol-amide(2) radical instead of a propanol-amide(2) group. It is 1.5 to 2 times more active than ergometrine, while possessing a similar uterine action. It acts within 20 to 60 sec. intravenously, 30 to 60 sec. intramurally, and 2 to 5 minutes intramuscularly; orally, it acts within 3 to 8 minutes. Its action is more prolonged than that of ergometrine but less than that of ergotamine. It is well tolerated even in high doses. It possesses no sympatholytic activity and is valueless in migraine. It is indicated in all uterine hæmorrhages, especially those due to post-partum atony, and in lochiometra; endometritis, incomplete abortion and Cæsarian section. It is contra-indicated before the birth of the child and is not recommended for the treatment of primary or secondary uterine inertia. It is supplied in bottles containing 10 ml. of a solution, 0.25 mg. in 1 ml., and in boxes of 6 ampoules 0.2 mg. in 1 ml.

S. L. W.

Neodrenal*, isopropyl adrenaline, is a stable synthetic sympathomimetic amine related to adrenaline. It is effective by mouth and is administered either as a sublingual tablet or as a spray-solution. It is employed for the symptomatic relief of bronchial asthma, spastic bronchitis, and status asthmaticus. The dose by sublingual administration is $\frac{1}{2}$ to $1\frac{1}{2}$ tablets (10 to 30 mg.) 3 times daily, gradually reducing to $\frac{1}{2}$ tablet daily; the effect is apparent within 4 to 10 minutes. Alternatively, relief may be obtained from oral inhalation of a 1 per cent. spray. Neodrenal is supplied in bottles of 25 or 250 tablets, each containing 20 mg., and in bottles of 15 or 100 ml. of a 1 per cent. spray-solution.

S. L. W.

a population could be divided into susceptible and immune persons: and Behring^{6,7} reported that mixtures of toxin and antitoxin could safely be injected into children. A certain mystery surrounded the manner of preparation and the composition of these early mixtures of T.A., as they came to be known, but it appears that the German workers were feeling their way and testing the antigenic efficiency, and the toxicity, of mixtures of different composition. Large numbers of children were inoculated in the Magdeburg district of Germany; the results were such that any remaining doubts in Park's mind regarding their safety were dispelled and, armed with this knowledge and with the Schick Test as a helpful guide, the way was clear for the application of these important advances in the United States. It is interesting to note that, while Park deprecated Behring's claim that these T.A. preparations were a new discovery, as they had been used extensively for the immunisation of animals, he emphasises the importance, to the campaign for active immunisation of man, of his demonstration of their safety.

Since its introduction the Schick Test has been the subject of much controversy, and the manner of its application, its interpretation, its value as an aid to diagnosis and as a guide for administrative action have all been challenged. These controversies cannot be discussed here, but the practical importance of the test at the time it was first applied can hardly be exaggerated. It gave confidence to field workers who were dealing with potentially dangerous materials of which they had little knowledge or experience; and, as these increased, the Schick Test became an almost essential part of the mechanism by means of which active immunisation of human populations has been achieved. One ground for criticism, however, has deserved more attention and consideration, in countries other than this, than it has actually received. Schick considered that the quantity of toxin contained in the Schick Test Dose was one-fiftieth of the minimum lethal dose of a matured toxin, as determined by injection into guinea-pigs. This definition is unacceptable on two grounds. Trevan⁸ pointed out that the term "minimum lethal dose" has no clear or proper meaning and indicates a vague quantity the actual determination of which is of doubtful accomplishment; and, even if it were permissible to define the Schick Test dose in terms of toxicity alone, the amount should be expressed in terms of the quantity found to be lethal for a stated percentage of animals as, for example, the LD₅₀ dose; this quantity has a meaning and can be determined with an accuracy dependent on the number of animals used. An even more serious objection to Schick's original definition was pointed out by Glenny⁹ and independently by Kellogg¹⁰: since the test determines the approximate concentration of antitoxin its definition must be expressed in terms of combining power for antitoxin and not only in terms of toxicity—the latter merely ensuring the presence of an adequate, but not too large, quantity of toxin for injection into human beings. The absurdity to which the test can be reduced by limiting its definition to toxicity was shown by Glenny and Waddington¹¹, who prepared a series

unprotected, the occurrence of cases and epidemics was a constant threat to life, especially of young children, and to the public health and economy.

Since it was known that susceptible animals could be successfully immunised against diphtheria there did not seem to be any reason why a similar result should not be achieved with man. The pathology of the disease indicated that the aim should be to provide protection against the characteristic toxæmia, and it is not surprising that the first attempts were on similar lines to those which had succeeded with animals, i.e., by the injection of toxin. Accordingly in the early years of this century there are a few records of the immunisation of man with small but increasing doses of toxin; the local reactions and the pain at the site of injection were reported. Attempts were also made to immunise man by the injection of bacterial vaccines. None of these measures, however, appeared very promising, and the success which has been achieved is a result of investigations on different lines.

In the United States, Theobald Smith^{1,2}, who then held important appointments at Harvard and the Antitoxin Laboratories of the Massachusetts State Board of Health, observed that the guinea-pigs supplied for his use were immune to diphtheria: on investigation he found that they, or their progenitors, had been used for testing diphtheria antitoxin, the injection of mixtures of toxin and antitoxin having served to immunise them. This observation was the starting point of a series of important researches concerning the properties and immunological behaviour of neutral, over-neutralised and under-neutralised mixtures in guinea-pigs and of the transmission of antitoxin, and in 1907 Theobald Smith advocated the use of certain of these mixtures for the active immunisation of man. It seems strange that it was not until 1913 that this suggestion, coming from so eminent and recognised an authority, was given practical application. The facts were not in question because many workers had shown that antitoxin was produced in animals which had survived the injection of mixtures of toxin and antitoxin. There appear to have been two main reasons for this delay; Park³, who was then and for many years afterwards the acknowledged leader in this field and was always ready to exploit any discovery or advance in the laboratory which promised to help in his fight against diphtheria in New York, described his own investigation of these mixtures in guinea-pigs. He observed that the mixtures which would have to be used for the effective immunisation of man were toxic for guinea-pigs and, quite clearly, Park was hesitant about injecting material into children which caused paralysis in guinea-pigs. Another deterrent was that, at that time, there was no simple means of finding out, first, which members of a community needed protection and, second, whether the measures taken had succeeded or failed.

In 1913, however, two papers appeared in Germany which removed these doubts. Schick⁴, basing his observations on the earlier work of Römer⁵, described his test by the application of which, it was claimed,

IMMUNISATION AGAINST DIPHTHERIA

Park and the early pioneers gave much thought and experiment to find the best mixture of T.A. for the immunisation of children. The balance of toxin and antitoxin had to be precisely adjusted: if the toxin were over-neutralised the mixture was not an effective antigen and if it were under-neutralised it might be unduly toxic and even dangerous. That the right balance had been struck was determined by injecting prescribed quantities into guinea-pigs, and thus the practice of ensuring the safety of prophylactics by tests on animals was begun. These have been developed so that tests for absence of specific and non-specific toxicity, antigenic activity, etc., have been incorporated in the official regulations and national pharmacopœias of many countries. Actually, T.A. was not easy to prepare and, of all the types of prophylactic which have been used, it gave the greatest anxiety. This was due to the fact that it contained toxin and, although this is neutralised with antitoxin and the preparation should be safe, it was a potentially dangerous substance and fatal accidents followed its use in several countries. Viewed in retrospect and in the light of what is now known of the way T.A. was made, the nature of the union of toxin and antitoxin and the circumstances under which they can dissociate, the remarkable thing is, not that there were a few accidents, but that there were not many more. In this country the authorities were so concerned at the possibility of accident that, for many years and until T.A. became obsolete, samples of every batch released for use were tested in the Department of Biological Standards at Hampstead for safety and antigenic activity.

It will be readily understood, therefore, that a milestone of very great importance in the development of diphtheria prophylactics was the discovery that the dangerous toxin could be replaced by harmless toxoid. In 1898 Salkowski¹⁷ observed that diphtheria toxin lost its toxicity when treated with formalin. Later workers have determined the conditions under which toxicity can be removed and immunising efficiency retained: in general it has been found that when incubated at 37°C. with a concentration of 0.5 per cent., or less, of formalin, diphtheria toxin loses its toxicity completely, usually in about four weeks, but it retains its combining power for antitoxin almost unchanged; and Lowenstein¹⁸, Glenny^{19,20,21} and Ramon^{22,23,24,25} showed that these non-toxic, sterile modifications of toxin—formol toxoid, anatoxin, or F.T. as they are now called—are effective antigens, Lowenstein's experiments relating to tetanus and Glenny's and Ramon's to diphtheria. Formol-toxoid is easy to prepare, it does not contain sensitising horse serum protein, it is quite devoid of specific toxicity and its specific reaction with antitoxin, *in vitro*, provides a ready method for its measurement by flocculation methods. In France it was used on a very large scale for the immunisation of children, and later of soldiers, and its success was such that it was widely used in other European countries and in North America. Its adoption in the United States followed the examination by Park and his colleagues²⁶ of samples sent to him by O'Brien and his colleagues, and the successful application of toxoids made in his own laboratory²⁷:

of Test Toxins, each conforming to Schick's original definition in that the dose, in each case, contained the amount of toxin postulated by Schick as the all important factor, but their combining powers varied to such an extent that they indicated antitoxin concentrations varying from one half of that usually detected by Schick Test Toxin to 200 times that level. It is difficult to understand why Schick Test Toxins should not be standardised on sound principles, based on a stable standard and thus reproducible and comparable the world over, especially as this could be done most economically, partly by means of the flocculation reaction and, in any case, by the use of a very few animals. This is not merely an academic question raising issues of no practical importance: how much of the controversies which have raged over the Schick Test in past years may have been due to faulty standardisation based on improper principles cannot be assessed; but the question is not without importance to problems which may arise in the future. It is quite likely that it may be necessary to estimate higher antitoxin concentrations in human populations than that hitherto indicated by the Schick Test Toxin as at present prepared for issue: this could be done without difficulty by selecting, or preparing, a Test Toxin having the desired combining power for antitoxin: its suitability for use, as indicated by this property and its content of free toxin, is easily checked by the intracutaneous injection of mixtures of the sample with antitoxin, and of a few dilutions of the preparation itself, into one or two guinea-pigs. The ease, convenience and economy are of less importance than the fact that the standardisation of the preparation is thus based on sound immunological principles.

Further progress was impeded in Germany by the first world war, but in America from 1913 onwards Park's^{12,13} energy and enthusiasm, his advocacy of the need for active immunisation, and his demonstration of the practicability and successful use of the T.A. preparations then available, provided a stimulus to workers in other countries and a model on which their efforts could be based.

1921 saw the beginnings of immunisation against diphtheria in England. In April, Glenny, Allen and O'Brien¹⁴ reported on active immunisation and the Schick Test. They outlined the measures which were practicable at that time and described the materials available. Later in the same year Copeman¹⁵ presented an important report on diphtheria to the Ministry of Health and described investigations at the Southmead Infirmary at Bristol; and in December, Copeman, O'Brien, Eagleton and Glenny¹⁶ gave a lucid account of how the Schick Test had been used to detect the susceptibles in a school at Mitcham, how 102 of these had been immunised with T.A., how all but two of these had become Schick-negative, which of them had shown local reaction and the character of those lesions. It is to be regretted that the recommendations of these early workers were not adopted as it is now recognised that many lives would thereby have been saved, much needless suffering avoided, and the conquest of diphtheria expedited.

IMMUNISATION AGAINST DIPHTHERIA

Park and the early pioneers gave much thought and experiment to find the best mixture of T.A. for the immunisation of children. The balance of toxin and antitoxin had to be precisely adjusted: if the toxin were over-neutralised the mixture was not an effective antigen and if it were under-neutralised it might be unduly toxic and even dangerous. That the right balance had been struck was determined by injecting prescribed quantities into guinea-pigs, and thus the practice of ensuring the safety of prophylactics by tests on animals was begun. These have been developed so that tests for absence of specific and non-specific toxicity, antigenic activity, etc., have been incorporated in the official regulations and national pharmacopœias of many countries. Actually, T.A. was not easy to prepare and, of all the types of prophylactic which have been used, it gave the greatest anxiety. This was due to the fact that it contained toxin and, although this is neutralised with antitoxin and the preparation should be safe, it was a potentially dangerous substance and fatal accidents followed its use in several countries. Viewed in retrospect and in the light of what is now known of the way T.A. was made, the nature of the union of toxin and antitoxin and the circumstances under which they can dissociate, the remarkable thing is, not that there were a few accidents, but that there were not many more. In this country the authorities were so concerned at the possibility of accident that, for many years and until T.A. became obsolete, samples of every batch released for use were tested in the Department of Biological Standards at Hampstead for safety and antigenic activity.

It will be readily understood, therefore, that a milestone of very great importance in the development of diphtheria prophylactics was the discovery that the dangerous toxin could be replaced by harmless toxoid. In 1898 Salkowski¹⁷ observed that diphtheria toxin lost its toxicity when treated with formalin. Later workers have determined the conditions under which toxicity can be removed and immunising efficiency retained: in general it has been found that when incubated at 37°C. with a concentration of 0.5 per cent., or less, of formalin, diphtheria toxin loses its toxicity completely, usually in about four weeks, but it retains its combining power for antitoxin almost unchanged; and Lowenstein¹⁸, Glenny^{19,20,21} and Ramon^{22,23,24,25} showed that these non-toxic, sterile modifications of toxin—formol toxoid, anatoxin, or F.T. as they are now called—are effective antigens. Lowenstein's experiments relating to tetanus and Glenny's and Ramon's to diphtheria. Formol-toxoid is easy to prepare, it does not contain sensitising horse serum protein, it is quite devoid of specific toxicity and its specific reaction with antitoxin, *in vitro*, provides a ready method for its measurement by flocculation methods. In France it was used on a very large scale for the immunisation of children, and later of soldiers, and its success was such that it was widely used in other European countries and in North America. Its adoption in the United States followed the examination by Park and his colleagues²⁶ of samples sent to him by O'Brien and his colleagues, and the successful application of toxoids made in his own laboratory²⁷:

an important factor in the substitution of F.T. for T.A. in North America in 1924 was the complete safety of the former and the rather frequent reminder that the latter might, on occasion, become toxic.

In this country, apart from its use during several years for the London County Council's immunisation campaign, formol-toxoid was not so extensively used as in other countries and one reason for this was that F.T. was liable to cause unpleasant local reactions, especially in adults: in this country this has always been regarded as a serious defect partly because of the adverse effect on the immunisation campaign. It is possible that this tendency to cause local reactions may have been due to the culture medium employed because formol-toxoid prepared in other countries, on the type of medium favoured by the French workers, appears to have been less objectionable in this respect. Another reason for the limited use of F.T. in England was that whereas, in many other countries these preparations were found to be so safe, so easy to prepare and control and so certain and regular in their behaviour, they were regarded as almost approaching the perfect prophylactic, in this country formol-toxoid has been regarded, not as the last word, but rather as the valuable raw material out of which more perfect antigens could be prepared.

It is interesting to note that it was in this country alone that the attempt was made to render T.A. safe by replacing the toxin by toxoid. Good service had been given by T.A. but it had now become possible to replace it by formol-toxoid, or improve it. Most countries adopted the former course, but in this country an improved and entirely safe type of prophylactic was developed from the original T.A. It was known that under-neutralised mixtures of toxin and antitoxin were excellent antigens, but they were toxic and might be dangerous; by using toxoid, or even toxin which had not been deprived of the last traces of toxicity, under-neutralised mixtures could be prepared, the antitoxin present being more than enough to neutralise the traces of toxin which might remain but insufficient to neutralise all the toxoid. These toxoid-antitoxin mixtures (T.A.M.)²⁸ were much easier to prepare than T.A. because precise adjustment of the two reagents was not so important, and some toxoid could be left unneutralised; they were widely used and retained their popularity long after more active, and in some ways more satisfactory, antigens became available. One instance of the successful use of T.A.M. was in Birmingham²⁹, where some 60,000 persons, mainly juvenile, were immunised: the fully detailed and documented record of this city's effort should be studied, for it demonstrated clearly that the disease could be held at bay, many hospital wards could be devoted to other uses and diphtheria brought under control at little cost; and it showed that the benefits which earlier workers had forecast would, indeed, be the reward of the sustained efforts they had called for ten years earlier.

A study of the properties and behaviour of the diphtheria prophylactics available at this time showed that some of them were prone to give

rise to local reactions on injection: and that different types of prophylactic produced their immunising effects in animals, and probably also in man, in different ways. Many observations suggested that the former phenomenon—local reaction—was associated with the non-specific impurities of the prophylactic, some being provided by the broth constituents on which the diphtheria organism was grown, others coming from the products of its metabolism and still others coming from the horse serum which provided the antitoxin of the preparation; and that the latter phenomenon—the mode of action—was associated with the size of the antigenic complex, the rate of its absorption and elimination, the long-continued stimulus which it exerted on the immunising mechanism and, perhaps, the slow dissociation of the combination, or slow release of antigen³⁰. Much information was gained concerning both these points by a study of the antigenic properties of the well-washed precipitates derived from neutral, under-neutralised and over-neutralised mixtures of toxin and antitoxin^{31,32}. It was shown that nearly the whole of the activity of a mixture is contained in the precipitate which separates from it, that the nitrogen content is remarkably low, that little activity remains in the supernatant fluid, and that the antigenic activity of the floccules, or precipitate, is directly related to the composition of the mixture from which it separates. The sterile emulsions of these toxin-antitoxin floccules is a highly purified form of prophylactic and one of the earliest to be prepared and, being free from non-specific impurities, it seldom gives rise to local reactions on injection. In a later series of experiments³³ the effect of the various impurities, removed during manufacture, on the antigenic activity of the highly purified floccule preparations which remained was determined.

The main results of these investigations were confirmed by Schmidt and Scholtz³⁴, and from their results and those of Eberhard³⁵ they considered that these washed toxin-antitoxin precipitates constituted the best antigens available at that time and they were used to a considerable extent in Germany; but, although available, they were not used in England. This was because the original floccule preparations contained toxin and, however firmly bound to antitoxin this may be, it does constitute a potential source of danger. In 1927, however, Glenny and Pope³⁶ showed that the toxin could with advantage be replaced by toxoid and that the resulting Toxoid-Antitoxin Floccules (T.A.F.) preparation is a highly effective antigen, possessing all the advantages of the similar precipitates prepared with toxin and is completely safe. T.A.F. quickly established itself as an effective antigen (Harries³⁷, Swyer³⁸, Freeman³⁹) and remains to-day, more than twenty years after its introduction, as one of the antigens of choice in this country. On the large scale it is prepared by allowing the precipitate which separates from under-neutralised mixtures of toxoid and antitoxin to sediment, washing the precipitate several times and finally emulsifying it in saline or other suitable solution in one-tenth, or less, of the volume of the original mixture. T.A.F. is characterised by the infrequency of local or general reactions following its injection, a

quality which makes it particularly suitable for the immunisation of adults, and by its efficacy as indicated by the high Schick conversion rate following injection. The fact that it contains horse serum and that three spaced injections must be given are drawbacks to its use.

The introduction of Alum Precipitated Toxoid (A.P.T.) is due to the work of Glenny^{40,41} and his collaborators. There was much evidence to show that increased antigenic activity was associated with the slow absorption and slow elimination of precipitated antigens⁴² and the behaviour of alum precipitated toxoid supported this view. The early preparations were used for the immunisation of animals and later Glenny and Barr⁴¹ emphasised the importance of removing the non-specific impurities from preparations intended for use on human beings. To this end Pope and Linggood⁴³ described the preparation of a culture medium of low nitrogen and controlled iron content which yielded high-grade toxins; the toxoid prepared from these, after purification with charcoal, is precipitated with a predetermined quantity of potash alum and the precipitate is further purified by treatment with disodium phosphate and saline. The antigenic value of the original toxoid is increased from fifty to a hundred fold by converting it into Alum Precipitated Toxoid⁴⁴.

Alum Precipitated Toxoid has advantages not possessed by other prophylactics which preceded it. It is a relatively pure substance, free from sensitising horse serum, slowly absorbed from the tissues and is not liable to cause local reactions especially after intramuscular injection; most important of all its advantages is that it is effective when administered in two small spaced doses while other prophylactics require to be administered by three injections. It is very certain in its effects; since 1941 several million persons, mainly juvenile, have received two injections and although it has not been possible to subject them all to the Schick Test, it has been shown in small communities which have been so tested that very high conversion rates followed such injections⁴⁵. Expert opinion is divided as to whether the Schick Test should be applied after a course of inoculations has been given, but more workers would agree that a case for its omission is more easily made out for A.P.T. than for any other type of prophylactic. It is claimed that by the correct use of preparations of A.P.T. of proved efficiency two visits to the clinic will suffice, instead of three, four and sometimes five, which may be required with other forms: this is an administrative convenience the importance of which should not be underrated.

With a view to improving still further the antigenic value of crude formol-toxoid Holt⁴⁶, at the Wright-Fleming Institute at St. Mary's Hospital, by the use of semi-synthetic medium in which the iron, salt and nitrogenous constituents were carefully adjusted, prepared high-grade toxins. The optimum conditions for their conversion into toxoid were established and then, by treatment with magnesium and cadmium compounds, followed by fractionation with ammonium sulphate, the large-scale production of purified toxoids of high activity was achieved. An

important development was the successful drying of these toxoids from the frozen state⁴⁷ and the optimum conditions for their adsorption on to pure aluminium phosphate. Bousfield⁴⁸ has reported very favourably on the clinical results obtained in the field. Tests on animals and human beings show that this latest form of prophylactic—P.T.A.P., as it is called—is a very active antigen and not liable to cause reactions after injection: the effect of the quantity of mineral constituents present in the completed antigen has also been investigated and some evidence has been obtained that the antigenic activity actually increases on storage, even under ordinary room-temperature conditions. A new point of great interest and of useful practical application is that, since large quantities of the highly purified toxoid can be prepared in the dry condition, and that the rest of the antigen is provided by pure inorganic salts added in a simple manner, the opportunity is exceptionally favourable for the preparation of successive batches of P.T.A.P. of uniform potency: tests of six successive batches on groups of 150 children confirmed this expectation⁴⁹. In view of the almost insuperable difficulties involved in the biological assay, by comparative tests in relation to the appropriate standards of the different types of diphtheria prophylactic employed in Great Britain, this possibility of ensuring a continued supply of antigen of proved efficacy is an important advance.

The different types of diphtheria prophylactic which research has provided for field use during the past thirty years form a logical sequence and, although the types used to-day are very different from those available to the early pioneers, each new arrival owes something to its predecessor. In the beginning the danger due to specific toxicity was a source of real anxiety and the factor which most limited progress for many years, but this was removed with the discovery and introduction of toxoid and then the question of local and general reactions claimed more attention. Active immunisation remains, however, a procedure involving the injection of small children, and the inclusion of the Schick Test increases the number of injections and adds other difficulties for the field worker; to any advance which reduces the number of injections must be assigned high merit. Accordingly, the object of research into the materials to be used for active immunisation has resolved itself into the search for a prophylactic which is free from all danger, which does not cause local or general reactions, which is certain in its action, which is effective after a minimum number of injections, which produces a high level of immunity without undue delay, and an immunity which is long-continued and permanent, and not transitory.

There has been steady progress towards the realisation of these aims, but new problems constantly arise. The question of the "injection of recall," or the "boosting dose" is more important than ever. Hitherto, the necessary stimulus to the immunising mechanism established by the injection of prophylactic could be looked for, in part at least, in the exposure of the immunised person to sub-clinical infection; but with the decline in the incidence of the disease this can no longer be counted

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upon. For this decline in this country we are indebted to the long-continued, many-sided labours of medical officers of health and others who have not only organised campaigns and acted as the instruments of national policy, but have actually immunised seven millions of our people: but while there are solid grounds for satisfaction, there are none for complacency and the effort must be maintained. In the absence of cases of diphtheria, as a reminder and a threat, it may not be so easy in future to maintain this high rate of primary immunisation, nor to convince the immunised adult that re-injection is necessary for his continued protection and safety. Epidemiologists and immunologists know that the present position of almost assured protection can so deteriorate that, with an inadequately immunised juvenile population and an adult population in which the immunity has declined, an extremely serious situation may develop; the reason for this may not be easy to explain to the layman, nor the reason why he cannot acquire protection against diphtheria as easily as when threatened with smallpox. Much thought, laboratory experiment and field trial will no doubt be given in order to reach a decision as to the best type of prophylactic to be used, the dosage, the route and the time for the re-injection of our young people all of whom, it is hoped, will have been fully inoculated before going to school. It does not necessarily follow that the type of antigen which succeeded so well in laying down a basal immunity is necessarily the best to use for the boosting dose or injection of recall, but there is now almost an embarrassment of choice of highly purified prophylactics available. The present indications are that it may be necessary to test each candidate for reinjection by the application of the Schick Test or some modified form of it, and to proceed in accordance with the result of that test. It will be interesting to see whether the increasing use of highly purified antigens affects the reaction of the individuals, and populations, to subsequent injections in later life.

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RESEARCH PAPERS

† AN EXAMINATION OF FACTORS AFFECTING THE PRECISION OF THE ASSAY OF THE OXYTOMIC HORMONE IN POSTERIOR PITUITARY LOBE PREPARATIONS

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EXCEPT in cases where partial separation of the oxytomic and pressor principles of posterior pituitary lobe have been made, this material is usually assayed by methods principally dependent on the oxytomic hormone. We have recently observed several instances of wide variation in the results obtained with the same sample of posterior pituitary lobe powder when assayed in different laboratories by the guinea-pig uterine method, and felt that a fairly comprehensive investigation of the slightly different techniques used might be of value.

Since the guinea-pig uterine method was originally described by Dale and Laidlaw¹, many workers have introduced minor modifications designed generally to overcome difficulties they have encountered themselves. Kochmann², for example, found that guinea-pigs weighing 200 to 250 g. were difficult to obtain in large numbers, and he therefore investigated the conditions under which the uteri from larger guinea-pigs could be used. He discovered that a decrease in the concentration of calcium chloride and an addition of magnesium chloride made possible the use of uteri from pregnant guinea-pigs, the weight of the animal being immaterial. Dale and Laidlaw¹, however, had previously stated that a rapid decline in responses to successive equal doses of posterior pituitary extract was shown when the calcium chloride was reduced to half the concentration in normal Ringer's solution. Burn and Dale³ reported that any method which involved the suppression of automatic rhythm by the reduction of calcium was unsuitable for their purpose. They insisted on the desirability of stating the composition of the Ringer's solution used when comparative results were presented, because changes in the composition of the physiological salt solution modified the sensitivity of the uterine muscle to pituitary extract, to histamine, and to potassium chloride, in varying degrees, and in different directions. This statement is important since many experimenters have looked for conditions under which the uterine assay may be performed more quickly and cheaply without considering the relative effects of their conditions on the response produced by the different hormones of the posterior pituitary gland. The principal modifications have been directed toward the elimination of the type of rhythmic response, occurring frequently, in which the uterus does not contract when stimulated by posterior pituitary extract, in proportion to the concentration of the drug in the bath.

THE INFLUENCE OF THE COMPOSITION OF THE RINGER'S SOLUTION

Apparent success in the development of a more reproducible response has followed modifications of the Ringer's solution in which the uterine muscle is bathed. The work of de Jalon⁴ showed that by decreasing the amount of calcium chloride in the Ringer's solution to 0.012 per cent. and by increasing the magnesium chloride to 0.029 per cent. responses were obtained in which the contraction was proportional to the concentration of posterior pituitary extract, whilst at the same time the sensitivity was increased. Hsu⁵ followed up the work of de Jalon and found that the assay of the oxytomic principle may be greatly improved by the use of 0.045 per cent. of magnesium chloride. He claimed that the muscle response was increased, that the spontaneous alternating rhythm of the uterine muscle could be broken or prevented, and that histamine did not interfere. A. M. Frazer⁶, however, was able to show that postlobin-V when measured against postlobin-O using Van Dyke and Hastings⁷ solution (a Ringer solution containing a large quantity of magnesium) had an oxytomic activity four times as great as when measured against postlobin-O in ordinary Ringer-Locke's solution, and that magnesium chloride increased the response of the guinea-pig's uterus to both postlobin-O and postlobin-V, the increase in response to the latter being much greater. This aspect of the assay technique was the first to be studied in these laboratories, since the results obtained in five different laboratories with a single sample of posterior pituitary lobe powder had ranged from 400 to 2,000 units/g., and it was known that there were differences in the saline solutions used throughout these comparative assays.

METHODS EMPLOYED

The usual isolated guinea-pig uterus preparation was used, the animals being virgin albino guinea-pigs weighing 200 to 250 g. The vessel containing the uterine muscle had a capacity of 20 ml. and no metal was in contact with the muscle or the solution, the uterus being impaled upon a glass spike at the bottom of the bath, and attached to the writing lever by a glass hook and a length of thread. The bath was aerated by compressed air, which we have always found to be satisfactory, instead of by oxygen as normally stipulated. Where normal Ringer's solution is referred to, the formula given by the British Pharmacopœia 1948 has been employed, and the modifications of salts to be described have been made to this basic solution. All records have been made using a 2× magnification by means of a light aluminium lever with frontal writing point, and a kymograph surface speed of 1 mm. per minute. Throughout the following experiments, only the magnesium chloride concentration has been modified, and the effect has been observed with mixtures made up from pitocin and pitressin (the purified oxytomic and pressor fractions prepared by Parke Davis and Co.) and with pitocin and pitressin alone. The dose/response relationship was determined for the sub-

maximal uterine responses to two different concentrations of the particular hormone, and the effect of adding different amounts of magnesium chloride solution prior to the addition of the hormones was examined; a section of an experiment is exemplified in Figure 1. The results were

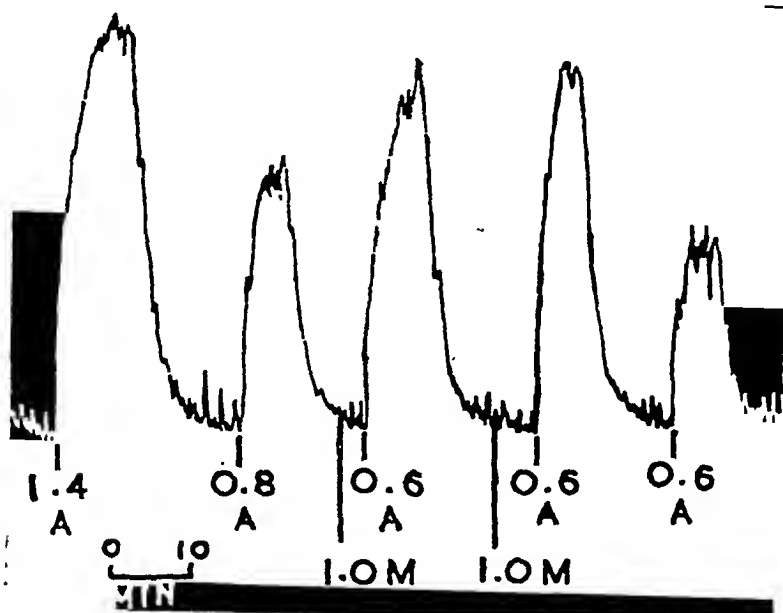


FIG. 1.—The influence of the addition of excess of magnesium chloride to Ringer's solution on the response of the isolated guinea-pig uterus to pitressin.

Doses of Pitressin in ml.

A = Pitressin, 20 pressor units per ml.; dilution 1:120.

1.0 M = 1.0 mg. of magnesium chloride.

expressed as the percentage increase in the response, and these values are shown in column 3 of Table I. A number of observations were made upon different uteri for each concentration of excess magnesium chloride. The results of Table I are expressed graphically in Figure 2, dotted vertical lines indicating percentage standard error, and show that in a concentration of 0.00125 per cent. of magnesium chloride in excess of that in the normal Ringer's solution, the potentiation effect both to pitocin and pitressin is practically nil, whilst at the excess concentrations of 0.0025 per cent. and 0.005 per cent. of magnesium chloride the response to both pitocin and pitressin is greatly enhanced, the potentiation of pitressin being much greater than that of pitocin. From 0.01 per cent. to 0.05 per cent. increase is observed for both hormones, although there is no significant difference between the influence of excess of magnesium chloride in either case. At a concentration of 0.1 per

ASSAY OF OXYTOMIC HORMONE

cent. of magnesium chloride in excess the potentiation of pitocin far exceeds that shown by pitressin.

In the main, the results support the work of Frazer¹, whereas his observations were made by introducing magnesium chloride into saline solution from which it was previously absent, the experiments described

TABLE I

THE INFLUENCE OF EXCESS OF MAGNESIUM CHLORIDE ON THE RESPONSE OF THE GUINEA-PIG UTERUS TO PITOCIN AND PITRESSIN

BASAL CONCENTRATION OF MAGNESIUM CHLORIDE IN RINGER SOLUTION
= 0.00025 per cent.

Excess Concentration of Magnesium Chloride	Number of Uteri	Increase in Response	
		Per cent.	Percentage Standard Error
		Pitocin	
0.00125	3	5	2.1
0.0025	4	43	4.4
0.005	2	82	2.2
0.01	6	70	26.6
0.025	4	45	24.8
0.05	8	46	24.8
0.1	2	187	3.0
		Pitressin	
0.00125	1	nil	
0.0025	2	129	9.0
0.005	3	104	10.6
0.01	2	44	10.0
0.025	3	59	18.1
0.05	4	95	30.5
0.1	2	76	2.0

above contained a minimum concentration of 0.00025 per cent. since uteri did not produce good contractions when stimulated with either pitocin or pitressin in a solution from which magnesium chloride was absent.

The second stage of the study of the effect of varying concentrations of magnesium ion comprised a series of assays of solutions with ratios of pitocin to pitressin from 4.0 down to 0.125 using the Ringer's solution proposed by (a) the British Pharmacopœia 1948 (magnesium chloride

TABLE II

TYPICAL DIFFERENCES IN GUINEA-PIG UTERINE ASSAY RESULTS USING TWO USUAL FORMULÆ FOR THE PHYSIOLOGICAL SALT SOLUTION

Ratio pitocin/pitressin	Actual total oxytocic potency U/ml. †	Ringer's solution B.P. 1948 potency found U/ml.	Ringer's solution with added Mg. (Hsu) [*]
4.00	10.1	8.0	10.6
2.00	10.2	9.2	10.6
1.00	10.4	10.0	10.6
0.50	10.8	9.3	13.6
0.25	11.6	11.2	13.6
0.125	13.2	13.0	19.2

* Hsu⁵ Ringer contains 0.045 per cent. of magnesium chloride.

† Allowance has been made for 4 per cent. of oxytocic hormone impurity in pitressin and for 4 per cent. of pressor hormone impurity in pitocin in computing the actual total oxytocic potencies of the solutions.

concentration 0.00025 per cent.), and (b) Hsu⁵ (magnesium chloride concentration 0.045 per cent.). The solutions were assayed against an extract of the International Posterior Pituitary Powder. The results are seen in Table II and show that the pressor principle exerts a significant oxytocic action when the ratio of pitocin to pitressin 0.50 or less,

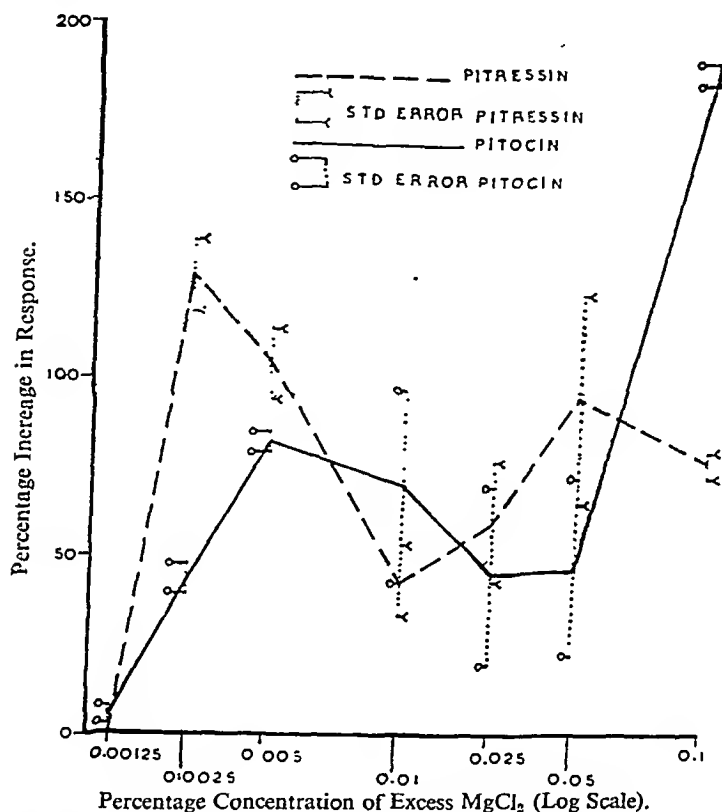


FIG. 2.—The effect of the addition of excess of magnesium chloride to Ringer's solution on the percentage increase in response of the isolated guinea-pig uterus to both pitocin and pitressin.

if Ringer's solution containing 0.045 per cent. of magnesium chloride is used. The potency found in Ringer's solution of the British Pharmacopœia 1948, for a mixture in which the ratio of pitocin to pitressin is 4.0 is significantly less than the actual potency, assuming a statistical limit of ± 20 per cent.

VARIATION OF CALCIUM ION CONCENTRATION

Since previous workers^{2,4}, claimed that physiological salt solutions with concentrations of calcium chloride different from that which is used in normal Ringer's solution have led to the improvement of uterine responses to posterior pituitary extract, the effect of varied concentrations of calcium

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chloride in the otherwise unchanged solution on the response of the uterus to pitocin and pitressin was studied next. The procedure followed was similar to that employed for the studies with excess of magnesium chloride. Figure 3 shows a section of a typical experiment in which the increase in the response to pitressin was studied after the addition of calcium chloride. Table III gives the results obtained, also expressed

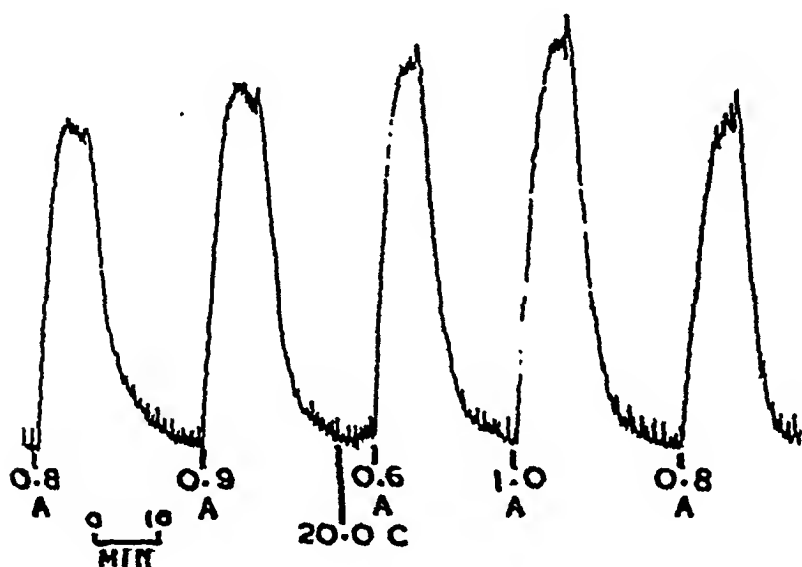


FIG. 3.—The influence of the addition of excess of calcium chloride to Ringer's solution on the response of the isolated guinea-pig uterus to pitressin.

Doses of Pitressin in ml.

A = Pitressin, 20 pressor units per ml.; dilution 1:120.

20.0 C = 20.0 mg. of calcium chloride.

graphically in Figure 4. At a concentration of 0.005 per cent. of calcium chloride in excess, pitocin shows a potentiation of 47 per cent. whilst there is no potentiation with pitressin; at concentrations of 0.01 per cent. and 0.025 per cent. in excess the potentiation with pitressin is greater than the potentiation with pitocin. There is no significant difference between the increase of pitocin and pitressin at a concentration of 0.05 per cent. of calcium chloride in excess, whilst at a concentration of 0.1 per cent. of the salt in excess it is the pitocin which shows the greater potentiation.

In the absence of calcium chloride from the Ringer's solution no response was given by the uterus to pitressin. The slight response that is shown in Figure 5 is probably due to a small amount of oxytocin as an impurity. Attempts to assay pitocin in the absence of calcium chloride were not successful. These observations on the effect of different concen-

TABLE III

THE INFLUENCE OF EXCESS OF CALCIUM CHLORIDE ON THE RESPONSE OF THE GUINEA-PIG UTERUS TO PITOCIN AND PITRESSIN

BASAL CONCENTRATION OF CALCIUM CHLORIDE IN RINGER'S SOLUTION
=0.024 per cent.

Excess Concentration of Calcium Chloride	Number of Uteri	Increase in Response	
		Per cent.	Percentage Standard Error
		Pitocin	
0.0025	3	2	1.0
0.005	2	47	1.0
0.01	2	18	5.0
0.025	4	18	7.0
0.05	5	52	8.6
0.1	2	104	9.8
		Pitressin	
		—20	11.5
		2	11.3
0.0025	7	35	4.9
0.005	3	37	12.4
0.01	3	38	9.4
0.025	4	62	12.5
0.05	4		
0.1	4		

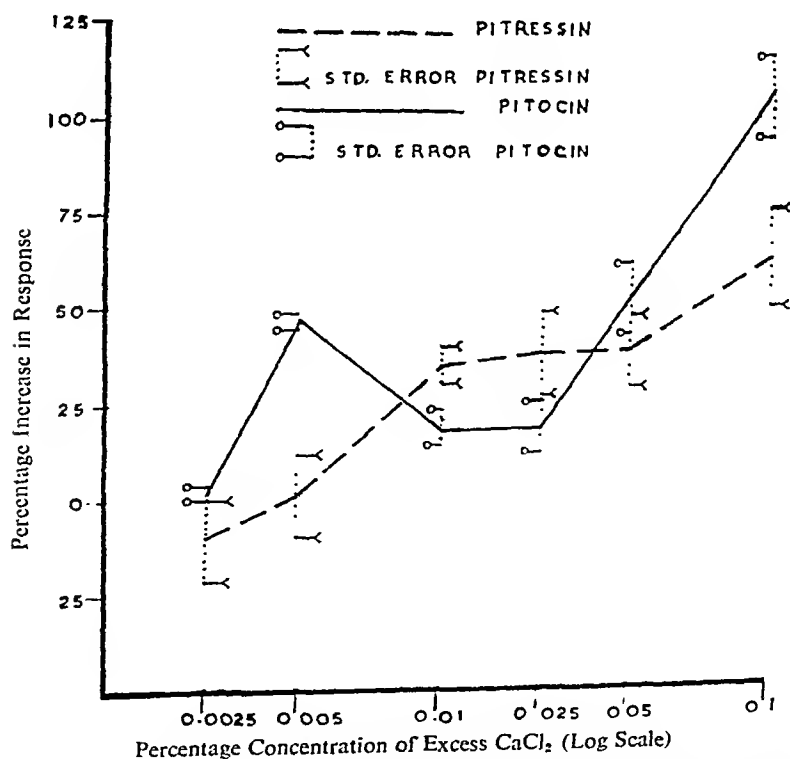


FIG. 4.—The effect of the addition of excess of calcium chloride to Ringer's solution on the percentage increase in response of the isolated guinea-pig uterus to both pitocin and pitressin.

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urines of both magnesium chloride and calcium chloride indicate that unless the ratio of oxytocin to vasopressin is unity, that is, the ratio in which oxytocin and vasopressin occur in the International Standard Posterior Pituitary powder, then the true oxytocic potency of the test material

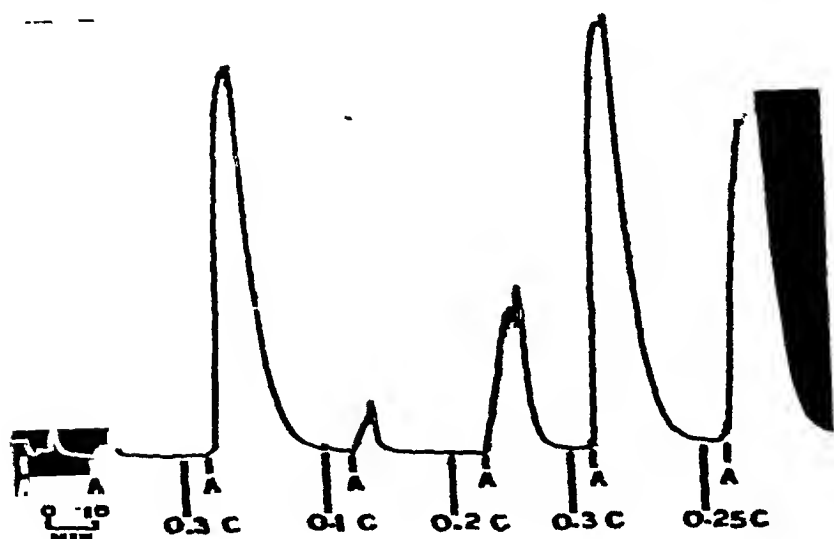


FIG. 5.—The influence of the addition of calcium chloride to Ringer's solution containing no calcium chloride on the response of the isolated guinea-pig uterus to pitressin.

A=0.3 ml. of Ringer's 20 pressor units per ml. solution 1:100.

C=Dose in mg. of calcium chloride.

cannot be calculated. Reference will be made later to work which has shown that this ratio can vary appreciably.

THE INFLUENCE OF DEXTROSE

Dextrose is a necessary constituent of the Ringer's solution, for in its absence the uterine muscle becomes rapidly fatigued by the first few doses of the posterior pituitary extracts, as Figure 6 indicates. In our experience dextrose at a concentration of that occurring in the normal Ringer's solution is sufficient to prevent the guinea-pig uterus from becoming fatigued for many hours.

THE EFFECT OF VARIABILITY OF THE UTERINE MUSCLE

Kochmann² and Merrill³ and his co-workers have described the assay of posterior pituitary extracts using the uteri from virgin guinea-pigs in various stages of the sexual cycle, and the former even used uteri from pregnant guinea-pigs. The following experiments were undertaken to ascertain what *diffusing* effect, if any, pitocin and pitressin produced on the uteri of guinea-pigs treated with progesterone and stilboestrol respectively. 1 mg./ml. of progesterone in arachis oil was administered subcutaneously in doses of 0.5 ml. per animal to a group of 6 virgin

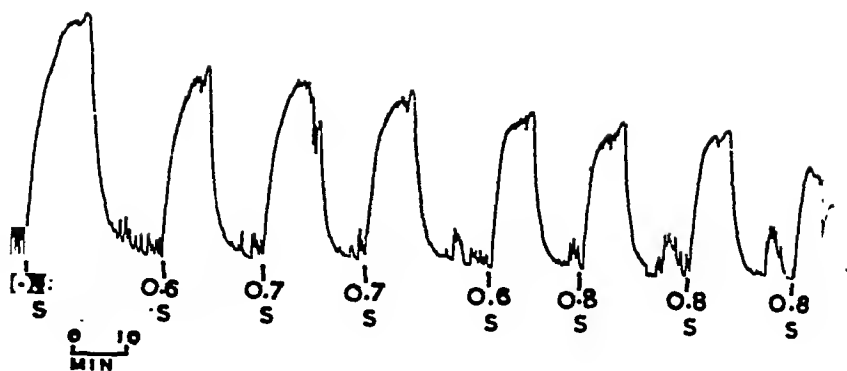


FIG. 6.—The response of the isolated guinea-pig uterus in Ringer's solution containing no dextrose to doses of pituitary extract.

S = ml. of Extract of International Posterior Pituitary Standard 2 I.U. per ml.; dilution 1:80.

albino guinea-pigs on each of 6 consecutive days. Stilbæstrol, 1 mg/ml. in arachis oil was given, at the same time, to another group of 6 virgin albino guinea-pigs each receiving 0.5 ml. subcutaneously on 6 occasions at intervals of 2 days. The concentration of drug at which the uterus just responded was taken as the limiting sensitivity of the uterus. The limits for pitocin and pitressin were observed, and the results are given in Table IV.

TABLE IV

THE SENSITIVITY TO PITOCIN AND PITRESSIN OF UTERI FROM VIRGIN GUINEA-PIGS TREATED WITH PROGESTERONE AND STILBÆSTROL

	Uterine Sensitivity		Sensitivity Ratio Pitocin/Pitressin
	Pitressin milliunits/ml	Pitocin milliunits/ml	
Progesterone-treated guinea-pigs	0.04 to 0.33	0.08 to 0.26	1.08
Stilbæstrol-treated guinea-pigs ...	0.01 to 0.04	0.002 to 0.018	2.50
Sensitivity ratio of progesterone-treated guinea-pigs to stilbæstrol-treated guinea-pigs ...	0.138	0.058	

The ratio of the sensitivity of pitocin and pitressin in the case of uteri from guinea-pigs treated with progesterone is 1.08, which indicates that pitressin exerts as great an oxytocic activity as pitocin, unit per unit, whilst in the case of the uteri from stilbæstrol-treated guinea-pigs the ratio is 2.50, pitocin thus exerting a greater oxytocic action than pitressin. The ratio of the sensitivity of the uteri from progesterone-treated guinea-pigs to those of stilbæstrol-treated ones, shows that in both cases the latter

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treatment produces the greater sensitivity, and this is even more marked with pitocin than with pitressin. The actual tracings showed that the response to posterior pituitary extract with the uteri from stilbæstrol-treated guinea-pigs were extremely rapid, reaching their maximal contraction in a few minutes, and also that there was no base rhythm (see Fig. 7). In the case of the uteri from guinea-pigs treated with

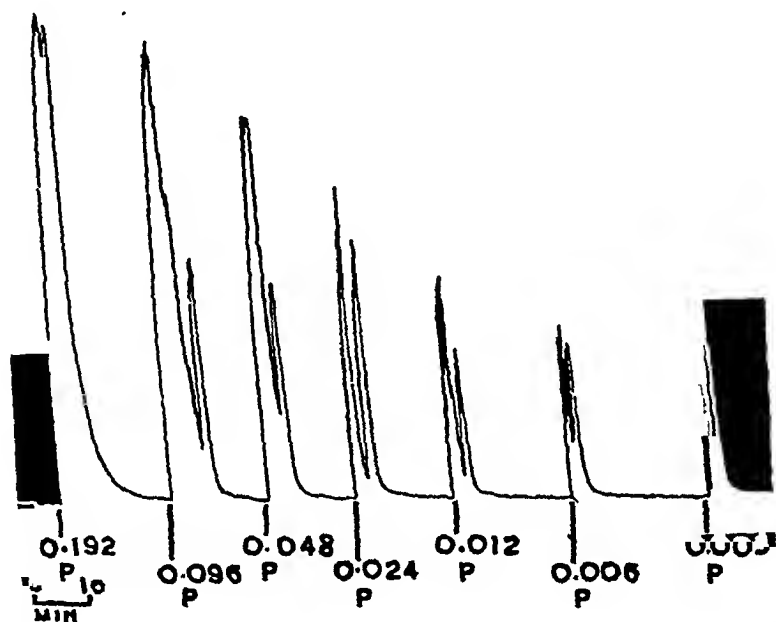


FIG. 7.—The response of the isolated uterus of a stilbæstrol-treated virgin guinea-pig to pitocin.

P = dose of pitocin in milliunits.

progesterone, however, the response to posterior pituitary extract was somewhat slower and the base rhythm was not abolished (see Fig. 8). Sufficient data were available to construct a histogram, shown in Figure 9, giving the average number of guinea-pigs, including failures, used for each assay every month during the period 1942 to 1946 inclusive. The blocks indicate the standard error. No one period of the year shows any very significant reduction in the number of guinea-pigs used per assay, though there is some indication that in July, August and September fewer guinea-pigs were used per assay than at other periods of the year, as indicated in the graph.

THE INFLUENCE OF TEMPERATURE

Hamburger⁹ has reported on the influence of changes in temperature of the Ringer's solution upon the response of guinea-pig uteri to posterior

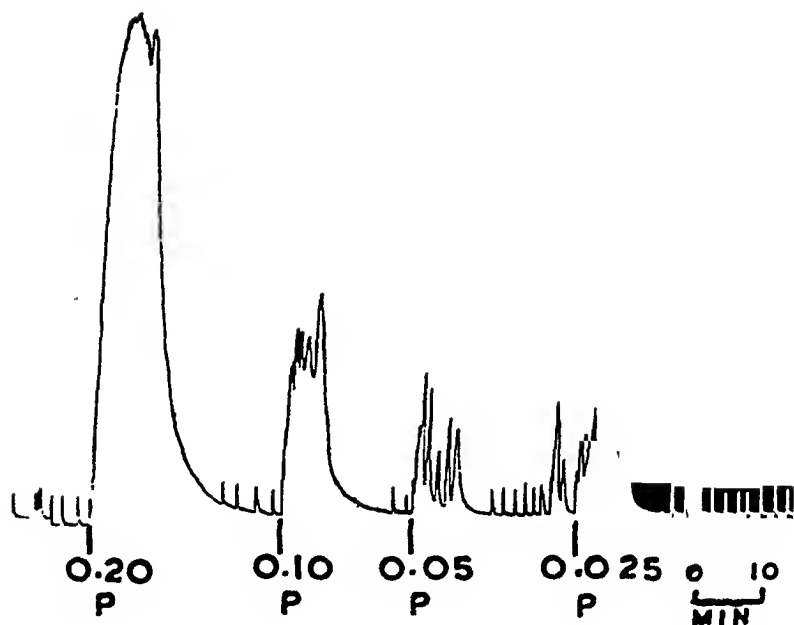


FIG. 8.—The response of the isolated uterus of a progesterone-treated virgin guinea-pig to pitocin.

P = dose of pitocin in milliunits.

pituitary extract. This effect we studied sufficiently to show that slight changes in temperature of the Ringer's solution do modify the response of the uterus to the hormonal extract, our observations being in close agreement with those of Hamburger. 37°C. is a suitable temperature at which to conduct the assay.

DISCUSSION OF UTERINE METHODS

Attempts have been made from time to time to establish an isolated uterine method of assay from which limits of error can be calculated. Hamburger¹⁰ modified the method of Burn and Dale³ and obtained for each guinea-pig uterus a number of contractions with posterior pituitary extract with doses ranging from that which produces maximum response to that producing minimum, and on the basis of the average height of the contractions for the individual doses plotted dose/response curves for the standard and unknown solutions. He introduced a dose into the bath every 10 minutes but in our experience this was not very practicable for rarely did the uterus relax before the introduction of the next dose into the bath. Introducing a dose immediately relaxation was achieved modified the subsequent response, but rhythm was not impaired.

Morrell⁸ *et al.* published a method based on a quantal response, and used the uterus of the virgin guinea-pig weighing 280 to 480 g. Regression lines were computed from which the error of assay could be

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calculated. They employed Van Dyke and Hastings' solution which contains 0.005 per cent. of calcium chloride and 0.009 per cent. of magnesium chloride. A later paper published by Bachinski¹¹ *et al.* stated that although the method using the quantal response possessed several advantages over the official uterine method it lacked the precision obtainable with a graded type of response. This paper sets out a uterine

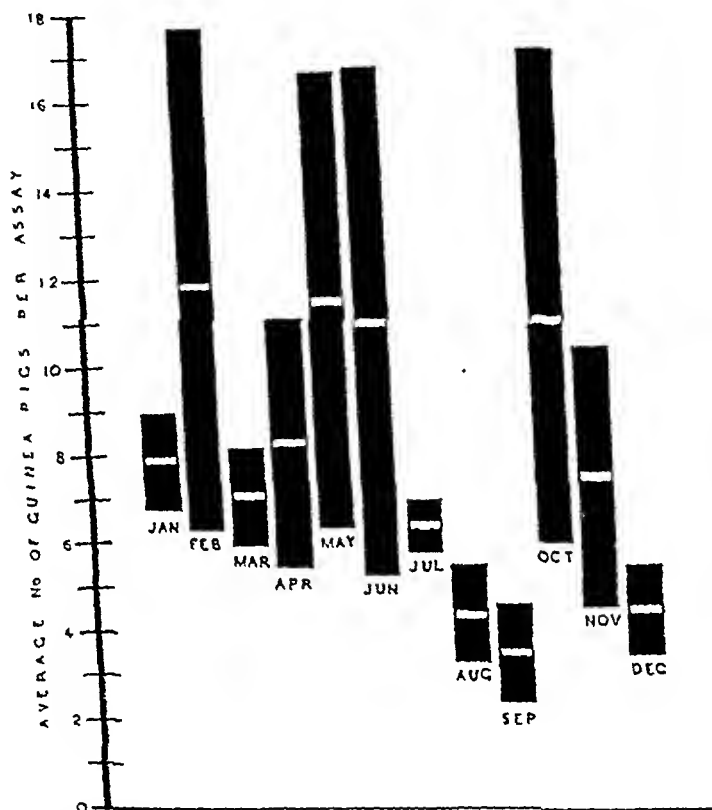


FIG. 9.—Histogram of the average number of virgin albino guinea-pigs used per assay for each month during the period 1942-46 inclusive. Narrow white strips indicate the mean values.

Blocks indicate the standard error.

method in which use is made of a quantitative response and yet the results stated in Table V of that paper show that 16 out of 23 results give percentage standard errors which do not include the true potency of the extracts assayed.

Holton¹² has recently described a precise and rapid method using the uterus of the rat maintained at 32°C. in a salt solution in which the calcium and glucose concentrations are respectively $\frac{1}{4}$ and $\frac{1}{2}$ that of Ringer's solution. Doses are randomised and limits of error are calculated. The mean percentage standard error is stated to be 2.16 from

8 satisfactory assays. The small limits of error have been confirmed in this laboratory though in one case they did not cover the true potency of a dilution of a commercial extract assayed against itself.

Of the four methods in use, three different modified Ringer's solutions are described, whilst the uteri from guinea-pigs in different stages of the sexual cycle have been used, and in one case the uterus from the rat.

Bachinski and Allmark¹³ studied the oxytocic activity of pitocin and pitressin on isolated segments of uteri from the guinea-pig, rat, rabbit and man. The muscle strips were excised at the normal, parturient, and postpartum states. A marked difference in response to pitocin and pitressin was observed. The uteri of the normal rabbit more nearly approached that of the "*in vitro*" parturient human uterus in the relative oxytocic activity of pitocin and pitressin in a given pituitary extract. They used Van Dyke and Hastings' solution since its magnesium chloride concentration approximates the serum level of the pregnant woman. Bachinski and Allmark¹³ claim that any indirect method such as the chicken depressor method may not agree with an assay using rabbit or human uterine strips. The difficulty encountered with any isolated uterine method, however, is the inability to make the method workable in different laboratories under exactly similar conditions, and a criterion of a method of standardisation must be that samples assayed in one laboratory shall be found to have the same potency, within statistical limits of error, as the same samples assayed in other laboratories.

Of the uterine methods the rat uterine assay is the most accurate since statistical limits can be computed, but the rat uterus is not similar to the parturient human uterus, according to Bachinski and Allmark¹³, and furthermore, in the light of the work which has been described in this paper there is reason to suspect that the rat uterus would be influenced by oxytocin and vasopressin to different degrees under variable conditions of assay. The factors involved are the variability of the different mammalian uteri to oxytocin and vasopressin, the sexual state of the uteri and the influence of one or all of the constituents of the physiological salt solutions in all their varying concentrations. Because of these factors the chicken depressor method for the assay of the oxytocic principle was examined.

THE CHICKEN DEPRESSOR METHOD

Coon¹⁴ gave a detailed account of the chicken depressor phenomenon and method of assay, and Thompson¹⁵ later refined and modified the method to give a very precise assay from which limits of error could be calculated. Although Thompson gives one result which differs significantly from the value obtained by the guinea-pig uterine assay, the latter appears to have been conducted on one uterine strip only, and it has been our experience, in the performance of many hundreds of uterine assays, that occasionally an answer produced consistently by one uterus for a given sample of posterior pituitary extract cannot be produced with subsequent uteri. Because of this, uterine assays have always been performed on a number of uteri. As it was impossible to obtain white

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Leghorn cockerels we used Rhode Island Red cockerels weighing 1.1 to 1.8 kg. The responses to pituitary extracts were very small indeed, and in two cases no response was produced at all. Light Sussex cockerels were in reasonable supply and these we finally adopted for the assay.

METHOD

The birds, weighing 1.8 to 2.3 kg. were anaesthetised by injecting phenobarbitone sodium 180 mg./kg. into the brachial vein. The popliteal artery and crural vein were dissected out and the blood pressure recorded from the former using a capillary mercury manometer. 8.5 per cent. of sodium citrate was used as anticoagulant. The International Standard Posterior Pituitary Extract, 2 oxytomic units per ml. was diluted 10 times with 0.9 per cent. saline solution and a volume not exceeding 0.4 ml. was injected rapidly into the crural vein. The doses were introduced into the vein at intervals of 3 to 5 minutes, but once the timing had been established it was strictly adhered to throughout the assay. A fall in blood pressure of 30 to 60 mm. of mercury was usually achieved. The unknown solution was diluted to a strength assumed to be equal to that of the Standard. Table V shows the results obtained in a comparison between the guinea-pig method of assay, using the Ringer's solution

TABLE V

COMPARISON OF THE GUINEA-PIG UTERUS AND CHICKEN BLOOD PRESSURE METHODS FOR THE MEASUREMENT OF OXYTOMIC ACTIVITY

Sample	Chicken depressor method U/g \pm S.E. (C)	Guinea-pig uterus U/g (G _p)	Ratio $\frac{C}{G_p}$
A	677 \pm 52	700	0.967
B	872 \pm 70	875	0.997
C	762 \pm 49	637	1.196
D	792 \pm 68	700	1.131
E	921 \pm 49	900	1.023
F	785 \pm 65	824	0.950
G	467 \pm 44	500	0.945

proposed by the British Pharmacopœia 1948, and the chicken depressor method as described above. The 4th column, with the ratios C/G_p, indicates the satisfactory agreement between the two methods of assay under the conditions stated.

Table VI gives the results obtained when mixtures of pitocin and pitressin were assayed. Pitressin exerted no oxytomic effect over a range of the ratio of pitocin to pitressin from 4.0 to 0.125. Coon found with white Leghorns that the ratio of oxytocin to vasopressin should not be less than 0.4 otherwise the vasopressin would influence the oxytomic response. The chicken depressor method has the added advantage over the guinea-pig uterine method is, that it is easy to perform and the pressor principle does not interfere with the ratio of pitocin to pitressin of 0.125. As many as 9 samples have been assayed on one bird in a day with 8 to 10 doses of the unknown per assay. As with the uterine assay, the results obtained with any one animal, however, should not be used unless confirmed at least once again on another bird.

As the guinea-pig uterine method is still the official method for assaying posterior pituitary extracts, this test is also performed in these laboratories, and confirmation has been obtained in 71 cases between

TABLE VI

THE INFLUENCE OF DIFFERENT RATIOS OF PITOCIN TO PITRESSIN ON THE ASSAY OF THE OXYTIC PRINCIPLE OF THE POSTERIOR PITUITARY LOBE GLAND BY THE CHICKEN DEPRESSOR METHOD

Ratio pitocin/pitressin	Actual total oxytocic potency U/ml*	Potency found	Limits of error (P = 0.95)
4.00	10.1	10.3	9.3 to 11.3
2.00	10.2	11.2	8.9 to 13.5
1.00	10.4	9.7	8.4 to 11.0
0.50	10.8	10.8	10.0 to 11.6
0.25	11.6	11.1	10.1 to 12.1
0.125	11.2	11.1	8.3 to 13.9

* Allowance has been made for 4 per cent. of oxytocic hormone impurity in pitressin and for 4 per cent. of pressor hormone impurity in pitocin in computing the actual total oxytocic potencies of the solutions

this method and the chicken depressor method. Furthermore, the chicken assay is a less costly test than the official method, and its greatest advantage lies in the fact that limits of error can be calculated and are of small magnitude.

THE STUDY OF COMMERCIAL POSTERIOR PITUITARY POWDERS AND EXTRACTS

Recent investigations into the large-scale extraction of posterior pituitary lobe powders revealed the fact that the oxytocic and pressor ratios in the extracts are not necessarily unity. In Table VII the results

TABLE VII

EXTRACTION OF COMMERCIAL SAMPLES OF POSTERIOR PITUITARY LOBE POWDER UNDER DIFFERENT CONDITIONS OF MANUFACTURE

Treatment	Pressor activity U/g	Oxytocic (depressor) activity U/g \pm Std error U/g	Ratio oxytocin/vasopressin
A	888	883 \pm 56	1.00
B	552	677 \pm 52	1.22
C	482	196 \pm 14	0.41
D	658	508 \pm 21	0.77

of four different extraction treatments are recorded. The ratio of oxytocin to vasopressin for treatment C is 0.41 and is significantly greater than that of any other. McClosky¹⁶ *et al.* has shown that in whale hypophysis also the ratio of vasopressin to oxytocin is greater than unity.

The method adopted for the assay of pressor activity was described by Kamm¹⁷ and his co-workers. Table VIII shows the results obtained in this laboratory for commercial extracts when assayed using the Ringer's solution proposed by the British Pharmacopœia. All the products

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were labelled 10 oxytocic units/ml. and their potencies range from 7.7 to 11.0 oxytocic units/ml.

Figure 10 is a tracing of an actual test, which is performed by obtaining two different submaximal responses to the extract of posterior pituitary standard. Next a dose of the unknown which gives a response intermediate between those of the two different standard responses is found. The three responses are repeated in a reverse sequence. Such a method shows whether or not rhythm has been introduced, in the latter

TABLE VIII

RESULTS OBTAINED FOR THE OXYTOCIC ACTIVITY OF COMMERCIAL POSTERIOR PITUITARY EXTRACTS, LABELLED TO CONFORM WITH THE B.P. REGULATIONS OF 10.0 OXYTOCIC UNITS PER ML., WHEN ASSAYED BY THE GUINEA-PIG UTERUS METHOD USING RINGER'S SOLUTION B.P. 1948

Sample	Potency U/ml	Limits U/ml*
A	10.0	8.6 to 11.4
B	8.8	7.5 to 10.0
C	7.5	6.7 to 8.9
D	11.0	10.0 to 12.2
E	8.8	7.5 to 10.0
F	7.8	6.7 to 8.9

* Range of upper and lower doses used in bracketing arrangement described for the uterine assay.

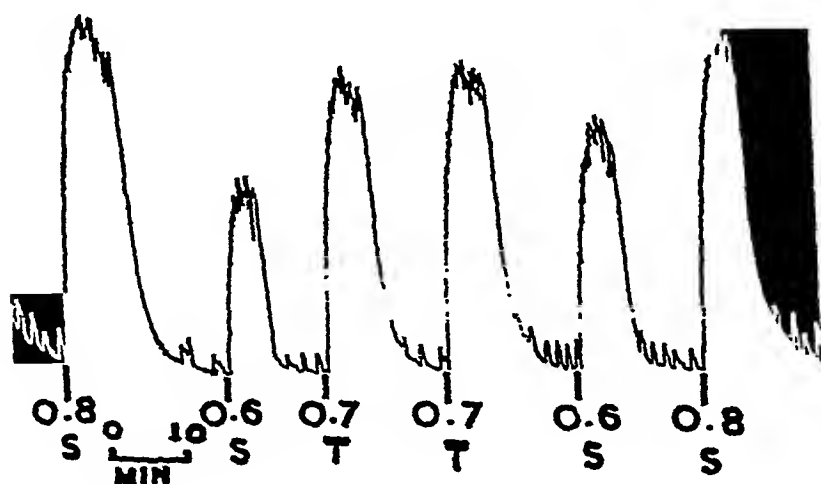


FIG 10—Typical example of the isolated guinea-pig uterine assay of a posterior pituitary extract.

S=ml of Extract of the International Posterior Pituitary Standard 2 I.U. per ml.; dilution 1:120.

T = ml. of Unknown, dilution 1:600.

case the test is discarded. At least two such complete tests are obtained from different uteri to constitute an assay.

SUMMARY

Work has been described which shows the errors which may be encountered under different experimental conditions of the uterine assay of oxytocin, or commercial posterior pituitary extracts where the ratio of oxytocin to vasopressin is not unity.

1. By increasing the concentration of magnesium chloride in the British Pharmacopœia 1948 Ringer's solution, the response of the guinea-pig uterus to pitocin is increased.
2. The response of the guinea-pig uterus to pitressin is also potentiated by increasing the concentration of magnesium chloride, but to a different extent than is pitocin.
3. Different concentrations of calcium chloride in the Ringer's solution produce potentiation to both pitocin and pitressin, but to different extents.
4. The pressor principle does not produce any oxytocic effect on the uterus suspended in Ringer's solution from which calcium chloride is absent.
5. The absence of glucose from the Ringer's solution produces rapid fatiguing of the uterine strip.
6. Temperature regulation of the Ringer's solution is very important.
7. The sensitivity of uteri from guinea-pigs treated with stilbœstrol is greater than the uteri of progesterone-treated guinea-pigs to both pitocin and pitressin.
8. The ratio of the sensitivity of uteri from progesterone-treated guinea-pigs to pitressin and pitocin is almost unity, whilst the ratio of sensitivity for the uteri of guinea-pigs treated with stilbœstrol indicates that the uteri are more sensitive to pitocin than to pitressin.
9. Seasonal variation in the number of virgin albino guinea-pigs used per assay was not found to be significant.
10. The ratio of oxytocin to vasopressin in the test samples must be the same as the ratio which is stated for the standard.
11. The type of uterus and the physiological salt solution should be stated.
12. It is recommended that the modification of Ringer's solution proposed by the British Pharmacopœia 1948 should be used for the guinea-pig assay, and that the uterus of the guinea-pig should be that of a virgin in diœstrus.
13. The chicken blood depressor method offers many advantages over the isolated guinea-pig uterine method. It is cheaper and quicker to perform, and is more accurate in that narrow fiducial limits can be obtained.
14. Until the chicken blood depressor method has been studied exhaustively in many laboratories engaged on the assay of the oxytocic

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principle, the guinea-pig uterine assay should be run in parallel with the former.

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THE ISOLATION OF BASES FROM URINE CONCENTRATES

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IN previous communications (Lockett^{1,2}) the name base B was applied to a compound present in the concentrates of steam distillate from normal dog and human urine, which had the property of raising the blood pressure of chloralosed cats, when injected intravenously. Concentrates of base B gave a non-specific colour test (x) when picric acid in chloroform solution was added to the base dissolved in a mixture of toluene and chloroform. Attempts to separate a compound responsible for colour production from that having pressor effect, or to demonstrate more than one compound responsible for either property failed. The methods employed were numerous, and included a long series of adsorption experiments, the use of many different precipitants and solvents; some of these experiments have been summarised (Lockett³). The two tests, colour production and pressor effect, were therefore considered to indicate the presence of a single compound which was shown to be volatile in steam, soluble in water, alcohol, chloroform, benzene and toluene, but to be insoluble in ether and light petroleum. It was unstable to acid and was readily oxidised.

In the first part of the present communication an account is given of the separation of base B of dog urine as the reineckate. The second part deals with the isolation of degradation products which were obtained from base B concentrates of human urine, and not from the reineckate. Two basic degradation products, piperidine and pyrrolidine, were obtained in pharmacologically significant amount, and had pressor activity. It was therefore of interest to compare the pharmacological actions of base B concentrates with those of these two degradation products. The pharmacological evidence (Lockett⁴) showed that the pressor activity of base B concentrates could be distinguished, in a number of ways, from that of piperidine and pyrrolidine; it therefore supported the chemical evidence that these last two bases were only obtained from base B concentrates by degradation.

METHODS

Preparation of base B concentrates. Two methods were used, and gave concentrates of approximately equal pressor activity and solid content per litre of original urine. The reineckate of base B separated more readily from concentrates prepared by method 2.

Method 1. This has been described under the heading of the preparation of concentrates of x-positive base (Lockett³). Nicotine was removed by adsorption on blood charcoal.

Method 2. The urine was brought to pH 11.6 (sodium hydroxide), and was submitted to continuous ether extraction for 24 to 30 hours. The extracted urine was distilled in steam, pH 12.0 (sodium hydroxide) and

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the distillate was concentrated *in vacuo*, below 35° C., without the addition of acid. No blood charcoal was used.

Separation of the reineckate of base B from dog urine. Base B concentrates were prepared by method 2 from successive 3 l. fractions of dog urine. Each concentrate (vol. 3.0 to 4.0 ml.) was neutralised to litmus as external indicator with N/1 sulphuric acid. A 4 per cent. solution of ammonium reineckate was added until no further precipitation occurred, and the solution remained well coloured. After standing in the ice-chest for 48 hours, the crude reineckate was collected, washed by centrifugation, and dried over calcium chloride; the collected reineckate was recrystallised from methyl alcohol.

The degradation of compounds present in base B concentrates from normal human urine. Incomplete oxidation with potassium permanganate in acid solution. Successive fractions of base B concentrates from 413 l. of normal human urine were treated with N/10 potassium permanganate in 1 per cent. sulphuric acid at room temperature, the oxidation being carried to a point just beyond the end of the rapid phase (2 to 3½ hours). The manganese dioxide was filtered off, and the filtrates were combined and distilled in steam from acid solution; the distillate gave a precipitate with silver nitrate, in the presence of nitric acid. After cooling the flask contents were extracted with ether; the ether extracts were dried over anhydrous sodium sulphate, the ether was removed by distillation, and there remained a small quantity of ether-soluble oil. The ether-extracted acid aqueous solution was freed of ether, cooled in ice, made alkaline with sodium hydroxide, and distilled in steam, dilute hydrochloric acid traps being used to prevent the escape of highly volatile bases. The distillate was acidified with hydrochloric acid, the trap contents were added, and the whole was evaporated to dryness under reduced pressure. The residue of crude hydrochloride was several times treated with alcohol and again evaporated to dryness. A search was made, without success, for non-volatile bases remaining in the flask contents after distillation from alkaline solution.

Complete oxidation with potassium permanganate in alkaline solution. The procedure differed little from that described for oxidation in acid solution. The concentrates were derived from 306 l. of urine. Oxidation with N/10 potassium permanganate in 2 per cent. sodium carbonate solution was carried to completion. The combined filtrates were acidified with sulphuric acid, and were worked up as described above under oxidation in acid solution.

RESULTS

Precipitation of base B as the reineckate. Neutral one-thousandfold concentrates of base B, prepared by method 2 from dog urine, yielded a crystalline powder when treated with ammonium reineckate. The crude reineckate had pressor activity, and its separation was attended by complete loss of pressor activity from the supernatant fluid (Fig. 1). Approximately 4 mg. of crude reineckate (chromium 14.1 per cent.) was obtained per l. of original urine. One-fifth of this weight represented

base. Since base B concentrates contained approximately 1 mg. of solid per l. of urine, there were other pharmacologically inert compounds present in base B concentrates which were not precipitated by ammonium reineckate.

Eight recrystallisations of the reineckate from 24 l. of dog urine yielded a compound which composed 90 per cent. by weight of the crude solid.

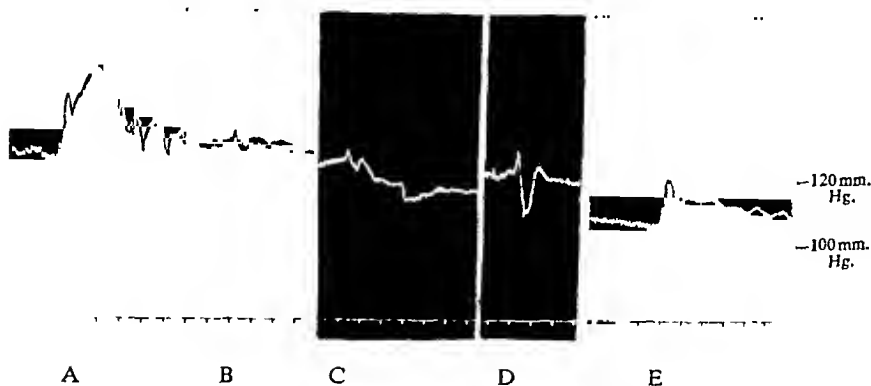


FIG. 1. Responses of the arterial blood pressure of a cat under chloralose anaesthesia, wt. 2.8 kg., to intravenous injection:—(A) 4.5 ml. of base B concentrate, equivalent to 0.7 l. of urine; (B) 5 ml. of the filtrate after the addition of 0.5 ml. of 4 per cent. ammonium reineckate solution to 4.5 ml. of base B concentrate; (C) 0.5 ml. of 4 per cent. ammonium reineckate solution diluted to 5 ml.; (D) 10 ml. of 10 per cent. ethyl alcohol; (E) 5 mg. of crude reineckate dissolved in 1 ml. of ethyl alcohol and diluted rapidly to 10 ml. with 0.9 per cent. sodium chloride solution just before injection. Each solution contained 0.9 per cent. of sodium chloride. Time marker, 30 sec.

The pressor activity of this material was less than that of the crude reineckate. Although five further recrystallisations did not significantly alter the analyses, the purified reineckate obtained at the end had only a depressant action on the blood pressure (Fig. 2).

Found: Crude reineckate Cr, 14.1 per cent.; 5th recrystallisation Cr, 13.8 per cent. 8th recrystallisation Cr, 13.9 per cent. 13th recrystallisation C, 22.31; H, 4.05; N, 26.8; Cr, 13.9 per cent. C_8H_9N requires C, 22.28; H, 4.14; N, 25.92; Cr, 13.76 per cent. $C_8H_{16}N_2$, dibasic, requires C, 22.28; H, 3.98; N, 25.99; Cr, 13.79 per cent.

The crystals from methyl alcohol were purple-red and grew in clusters. They were almost totally insoluble in water.

The isolation of degradation products from base B concentrates. The study, by degradation, of compounds present in base B concentrates, prepared either by the first or second method, from human urine, has consistently yielded simple volatile bases which can readily be separated in crystalline form. Before degradation these bases could not be crystallised from base B concentrates. The bases isolated after degradation were ammonia, dimethylamine, piperidine, pyrrolidine, and two unidentified compounds.

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Several volatile pressor bases have already been isolated from normal urine:—nicotine (Dingemans and Freud⁵), isoamylamine (Bain⁶), a nicotine compound (Lockett¹), piperidine (Euler⁷). These compounds

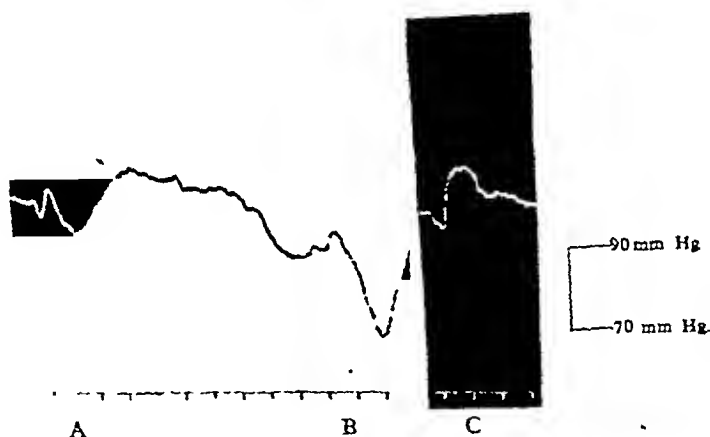


FIG. 2. Responses of the arterial blood pressure of a cat under chloralose anaesthesia, wt. 2.1 kg., to 4 mg. of crystals dissolved in 1 ml. of ethyl alcohol, and diluted to 10 ml. with 0.9 per cent. sodium chloride solution immediately before injection:—(A) 8th recrystallisation of the crude reineckate; (B) 13th recrystallisation of the crude reineckate; (C) crude reineckate. Time marker, 30 sec.

are soluble in ether and would therefore have been extracted from concentrates prepared by method 2. Using concentrates prepared by each method, in six experiments, the addition of piperidine, dimethylamine, and ammonia in concentrations varying from 1 in 1000 to 1 in 10,000, and of nicotine in a concentration of 1 in 20,000 to measured aliquots of the original urine, yielded concentrates which did not differ significantly in pressor activity from concentrates obtained from untreated aliquots of the same urine. In no case could the compound be recovered from the treated concentrates. The added bases had therefore been removed by the processes involved in the preparation of base B concentrates by either method.

Incomplete oxidation of base B concentrates in chlorine-free sulphuric acid solution yielded a mixture of bases, an ether-soluble oil, and hydrochloric acid. The ether-soluble oil had a pungent smell, and some volatility in ether vapour; it soon deposited floccules on standing; the addition of alcohol to its ether solution resulted in a white floccular precipitate, insoluble in water, acids, alkalis, chloroform, but with a very low solubility in ether. After drying in a high vacuum at room temperature the compound was white and waxy; sodium fusion showed the presence of C and H, and absence of N, halogen, P and S. Found C, 84.16; H, 13.19 per cent.; mol. wt. by depression of the freezing-point of camphor, 940.

The crude hydrochloride, which contained all the bases, was separated into alcohol-soluble and alcohol-insoluble fractions. The alcohol-insoluble hydrochloride, after recrystallisation, was white, mildly deliquescent, and decomposed slowly with the appearance of sublimation from about 280°C. The whole was converted to picrate, and was purified by recrystallisation. The base was liberated from the pure picrate and a picrolonate, nitrate, phosphomolybdate, and platinichloride were prepared. The analyses and melting-points corresponded closely with those of ammonia derivatives, and the mixed melting-points were satisfactory. This base formed nearly three quarters of the total weight of crude hydrochloride obtained.

1. *Identification of ammonia*.—Picrate from alcohol-insoluble hydrochloride, yellow needles, m.pt. 291°C. Ammonium picrate similarly prepared, m.pt. 291°C. with decomposition in each case. Found: C, 29.77; H, 2.39; N, 22.6 per cent. Calculated: C, 29.27; H, 2.44; N, 22.77 per cent.

Picrolonate. Purple sheen. Decomposed 280°C. Ammonium picrolonate had the same appearance, and decomposed at 280°C. Found: C, 43.38; H, 4.1; N, 25.3 per cent. Calculated: C, 42.71; H, 3.9; N, 24.9 per cent.

Nitrate. Transparent needles, m.pt. 168°C. Ammonium nitrate m.pt. 169°C., mixed m.pt. 168°C.

The crude alcohol-soluble hydrochloride was strongly deliquescent; the whole was converted to picrate, and fractional crystallisation was carried out. Great difficulty was encountered from oil formation, and the separation was not quantitative; five well defined fractions were obtained and recrystallised to constant melting-points. From three of these picrates other derivatives were prepared which led to their identification as piperidine, pyrrolidine, and dimethylamine. The fourth and fifth picrates were obtained in rather small amount, 25 and 16 mg.; they have not yet been identified.

2. *Identification of piperidine*. Picrate m.pt. 145°C. Piperidine picrate m.pt. 147°C. Mixed m.pt. 145°C. Found: C, 41.55; H, 4.1; N, 17.9 per cent. C, 41.73; H, 4.57; N, 18.1 per cent. previous experiment. C, 41.96; H, 4.52; N, 18.3 per cent. previous experiment. Calculated: C, 42.04; H, 4.46; N, 18.1 per cent.

Picrolonate. Brownish yellow, m.pt. 248°C. with decomposition. Piperidine picrolonate decomposed 248° to 250°C. Found: C, 51.47; H, 5.3; N, 21.6; N-Me. 0 per cent. C, 51.2; H, 5.16; N, 21.0; N-Me. 0 per cent. previous experiment. C, 50.05; H, 5.48; N, 19.6 per cent. previous experiment. Calculated: C, 51.58; H, 5.45; N, 20.01 per cent.

Platinichloride, from aqueous solution, m.pt. 201°C. Piperidine platinichloride m.pt. 201°C. Mixed m.pt. 201°C.d.

3. *Identification of dimethylamine* Picrate m.pt. 155°C., yellow flattened plates. Dimethylamine picrate m.pt. 156°C. Mixed m.pt. 156°C. Found: C, 35.69; H, 3.12; N, 21.15 per cent. C, 35.2; H, 3.4; N, 20.8 per

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cent: previous experiment. Calculated: C, 35.3; H, 3.6; N, 20.5 per cent.

Platinichloride. Found: C, 9.32; H, 2.28; N, 6.22 per cent. Calculated: C, 9.6; H, 3.2; N, 5.8 per cent.

Picrolonate, yellow, m.pt. 252°C. Dimethylamine picrolonate m.pt. 252°C. Mixed m.pt. 252°C., each with decomposition.

4. *Identification of Pyrrolidine.* Picrate m.pt. 112°C. Pyrrolidine picrate m.pt. 112°C. Mixed m.pt. 112°C. Found: C, 40.39; H, 5.04; N, 18.0 per cent. Calculated: C, 40.0; H, 4.0; N, 18.67 per cent.

Picrolonate, brick red, decomposed from 260°C. to 264°C. Found: C, 50.62; H, 5.25; N, 21.0; N-Me, 0 per cent. Calculated: C, 50.16; H, 5.08; N, 20.9 per cent.

Gold chloride, m.pt. 206°C.d. Pyrrolidine 206°C.d.

5. *Data for unidentified base.* Picrate, pale yellow needles, m.pt. 136°C. Found: C, 41.2; H, 4.26; N, 18.5; N-Me, 8.8 per cent. Calculated for $C_9H_{20}N_2$, and 2N-Me, dibasic, C, 41.04; H, 4.24; N, 18.24; N-Me, 9.4 per cent.

Picrolonate, yellow orange, decomposing 258°C. to 263°C. Found: C, 50.67; H, 5.6; N, 21.4 per cent. Calculated: C, 50.88; H, 5.26; N, 20.46 per cent.

6. *Data for unidentified base.* Picrate, golden needles, decomposing at 281°C. Found: C, 42.47; H, 4.33; N, 18.1, 18.9; N-Me, 6.8 per cent.

Oxidation of base B concentrate by N/10 potassium permanganate in 2 per cent. sodium carbonate solution, when carried to completion, yielded hydrochloric acid, and two volatile bases. The first base was present in large amount, and was identified as ammonia; the second, isolated as a picrate which was eventually crystallised, was present in small amount, and was identified as dimethylamine.

7. *Identification of Ammonia.* Picrate, long yellow needles, m.pt. 291°C. Mixed with ammonium picrate, m.pt. 291°C. Found: C, 29.55; H, 2.48; N, 23.0 per cent. Calculated: C, 29.27; H, 2.44; N, 22.77 per cent.

Picrolonate, purple sheen, decomposing at 280°C. Resembled ammonium picrolonate both in appearance and in decomposition point.

Acetate, m.pt. 87°C. Mixed m.pt. 87°C.

Phosphomolybdate. Greenish yellow crystalline powder.

8. *Identification of dimethylamine.* Picrate, m.pt. 155°C. Mixed with dimethylamine picrate m.pt. 155°C

9. *Identification of chlorine.* The precipitate with silver nitrate solution in the presence of nitric acid, was washed with water and alcohol, and was dried *in vacuo*, and was analysed. Found: Cl., 25.2 per cent. AgCl requires Cl, 24.9 per cent.

Pressor activity of pyrrolidine and piperidine. Since piperidine and pyrrolidine both have pressor activity, the identities of picrates 2 and 4 were further established by comparison with those of piperidine and pyrrolidine in respect of their effect on the blood pressure of a cat under

chloralose anaesthesia. In equating these picrates it was found necessary to wait 15 minutes between each injection, since repeated injections of pyrrolidine, in the same dose, at short intervals, sometimes produced decreasing responses.

7.2 mg. of picrate 2 equated very nearly with 7.0 mg. of piperidine picrate, both given in a volume of 3.0 ml. when similarly administered to a cat, wt. 2.4 kg. (Fig. 3A and B), 4.6 mg. of pyrrolidine picrate gave

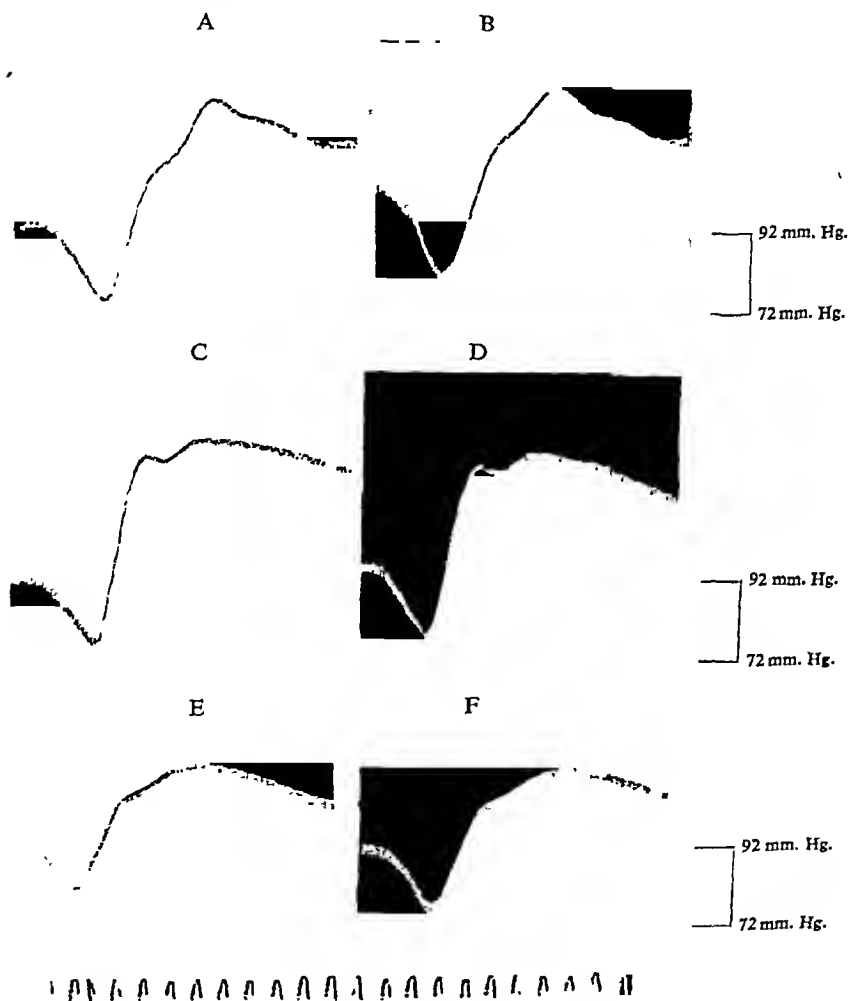


FIG. 3. Responses of the arterial blood pressure of a cat under chloralose anaesthesia, wt. 3.6 kg., to intravenous injection:—(A) 7.2 mg. of picrate 2; (B) 7.0 mg. of piperidine picrate; (C) 4.6 mg. and (D) 4.2 mg. of pyrrolidine picrate; (E) 4.5 mg. and (F) 4.1 mg. of picrate 4. Time marker, 30 secs.

a response comparable with that which followed 4.5 mg. of picrate 4, each in a volume of 4.5 ml. (Fig. 3C and D) and 4.2 mg. of pyrrolidine

picrate equated with 4.1 mg. of picrate 4, each in a volume of 4.0 ml. (Fig. 3E and F).

DISCUSSION

The interest of these results is centred on the high degree of pressor activity which must be attributed to base B. Since the purified reineckate accounted for at least 90 per cent. of the crude solid, and 5 mg. of crude material were obtained per l. of original urine, 0.5 mg./l. of urine is the maximum weight that could be ascribed to base B. Of this 0.5 mg. it is unlikely that more than 20 per cent. by weight could have represented free base. The weight of base B required to produce a small or moderate rise of arterial blood pressure when injected intravenously into a cat, cannot be greater than 100 μ g. That the estimated dose may prove to be considerably greater than the real is suggested by the results recorded in Figure 2, and the analyses listed; in the absence of any change in the chromium content, pressor activity was succeeded by depressor action as the result of repeated recrystallisation. The depressor compound isolated has been found to give the colour test x which previously appeared to be inseparable from the pressor action of base B concentrates, and was therefore used as an aid to the separation of base B. This depressor compound may prove to be a simple diamine; its pharmacological action is compatible with this view.

The occurrence of piperidine and pyrrolidine as degradation products of compounds present in base B concentrates is interesting, and indicates that further evidence relating to the intermediary metabolism of these heterocyclic compounds may be obtained from a study of urinary trace bases.

SUMMARY

1. Base B has shown to be a compound of high pressure activity, and a depressor compound, empirical formula $C_6H_{16}N_2$ has been isolated as the reineckate from base B concentrates.

2. Piperidine, pyrrolidine, dimethylamine and ammonia have been obtained as degradation products from compounds present in base B concentrates, prepared from human urine.

A grant from the Medical Research Council defrayed part of the expense of this work.

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THE IDENTIFICATION OF LOCAL ANÆSTHETIC DRUGS OF THE BENZOIC ESTER GROUP

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From the Metropolitan Police Laboratory

Received May 20, 1949

THE property of paralysing sensory nerve endings and inducing local anæsthesia is not confined to any one specific chemical structure. Indeed, from a chemical standpoint it is convenient to arrange local anæsthetics in three groups: (1) consisting mainly of hydroxy compounds (e.g. phenol, menthol) used largely for topical anæsthesia, (2) the benzoic ester group, and (3) various miscellaneous compounds of synthetic origin, and of widely differing chemical structure (e.g., cinchocaine, phenacaine).

By far the largest, and clinically the most important of these groups is that composed of the benzoic esters. Following the demonstration by Koller in 1884 that cocaine, methylecgonine benzoate, is an effective local anæsthetic for ophthalmic purposes, a wide range of benzoic esters has been synthesised and investigated as "cocaine substitutes." Whilst these are, for the most part, less toxic than cocaine itself, and are devoid of the latter's addiction liability, the relative toxicities depend largely upon the manner and rate of administration. At the present time, eleven benzoic ester local anæsthetics are in common use in this country (Table I), and with a single exception (butyl-*p*-aminobenzoate) are characterised by names ending in "-caine." This terminology, although admittedly useful in drawing attention to the local anæsthetic nature of a particular drug, has unfortunately been responsible on occasion for confusion during dispensing and use. Fatal poisonings with these drugs are usually the result of accident due to such confusion, to over-dosage, to intravenous instead of subcutaneous injection, or to individual hypersensitivity; suicidal poisonings are relatively rare.

Information as to the metabolic fate of these drugs is incomplete. Detoxification occurs largely in the liver with hydrolysis of the ester linkage, but there is little doubt that other tissues also play some part in the destruction of the drugs *in vivo*. In the case of cocaine, hydrolysis yields benzoic acid and ecgonine; elimination is relatively slow and the hydrolysis products, together with a proportion of unchanged cocaine, may be excreted *via* the kidneys over a period of several days. With the other local anæsthetics breakdown is, in general, a rapid process, and elimination as acetylated *p*-aminobenzoic acid is usually complete within a few hours. For these reasons, detection of unchanged drugs other than cocaine in cases of fatal poisoning is unusual, except where considerable doses have been administered, and attempts at recovery from cadaveric material after an interval of days are likely to be unsuccessful. Particularly important, however, is the examination of the liver, kidneys and urine, since, although insufficient unchanged drug may be obtained for purposes of identification, products of hydrolysis such as ecgonine and *p*-aminobenzoic acid may be recoverable.

IDENTIFICATION OF LOCAL ANÆSTHETICS

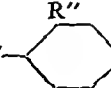
In view of the accidental character of most cases of local anæsthetic poisoning, the toxicologist's problem is seldom the identification of a completely unknown poison, but is usually the confirmation of the presence of a particular local anæsthetic drug. The most successful of the numerous methods hitherto described for differentiating and identifying the drugs of this group is probably that of Fischer¹, who utilises the crystalline forms and melting-points of derivatives formed with the reagents trinitroresorcinol, trinitrobenzoic acid, pieric acid and platinum chloride. There appears, however, to be no recorded comprehensive scheme whereby, on a micro scale, an unknown isolated residue may be characterised as a benzoic ester local anæsthetic, and subsequently completely identified.

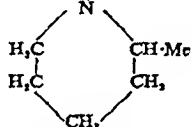
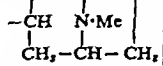
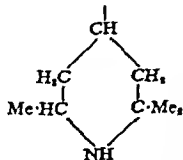
In the present investigation, dealing with the benzoic ester anæsthetics listed in Table I, a residue isolated either from biological material or from a medicinal preparation is examined in a series of separate stages: (1) purification of the crude residue, (2) behaviour with "alkaloidal reagents," (3) demonstration of benzoic ester structure, (4) simple crystal and colour reactions, (5) conclusive identification by micro mixed melting-point determination. It will be evident that the requirements of a particular problem may frequently be met without the inclusion of all these stages, subject to the use, whenever quantity permits, of the mixed melting-point as the final criterion of identity. The tests described are simple techniques, requiring a minimum of time, and employing only readily available reagents and apparatus. With quantities of the order of 1 mg., the scheme provides for the characterisation of an unknown drug as a benzoic ester local anæsthetic, and for its ultimate identification.

Isolation of local anæsthetic drugs. The basic character of the local anæsthetics of the benzoic ester group is such that, in general, they may be extracted with chloroform from solutions alkaline with sodium hydroxide, and thus in the Stas-Otto process are largely obtained in the non-phenolic alkaloid group. At the same time, hydrolysis may occur under the influence of the sodium hydroxide, and the yield of drug may in this way be appreciably reduced. Noteworthy exceptions are the structurally similar benzocaine and butyl *p*-aminobenzoate, which are extracted by ether from aqueous acid solutions, and are, therefore, obtained in the acid-ether extract during the normal Stas-Otto process. In the case of butyl *p*-aminobenzoate, hydrochloric acid is preferable to sulphuric acid for the preparation of the aqueous acid solution owing to the relatively insoluble nature of the sulphate of this drug. The phenolic drug orthocaine undergoes progressive decomposition in the presence of alkali, and dark-coloured residues are obtained in both the non-phenolic and phenolic alkaloid groups following the Stas-Otto procedure. This drug is best extracted with ether from aqueous solutions acidified with tartaric acid.

Purification of crude residues. When isolated from biological sources the drugs are invariably contaminated with brownish, often gummy, extractives. Since ether solutions of the free bases deposit the white hydrochlorides on treating with a stream of dry hydrogen chloride, this

TABLE I

LOCAL ANÆSTHETIC DRUGS (R'——COOR)

	R	R'	R''	Mp. (°C) of hydrochloride
AMETHOCAINE HYDROCHLORIDE B.P. ... Anethaine Butethanol Decicaine Pantocaine Tetracaine	—CH ₂ ·CH ₂ ·NMe ₂	C ₆ H ₅ ·NH—	H—	149
BUTACAINE SULPHATE B.P. Butyn	—CH ₂ ·CH ₂ ·CH ₂ ·NBu ₂	NH ₂ —	H—	151
PROCAINE HYDROCHLORIDE B.P. Ethocaine Kerocaine Novocaine Planocaine Seriacaine	—CH ₂ ·CH ₂ ·NEt ₂	NH ₂ —	H—	155
AMYDRICAINE HYDROCHLORIDE B.P.C. ... Alypin	—C Et (CH ₂ NMe ₂) ₂	H—	H—	169
METYCAINE (HYDROCHLORIDE) ...	—CH ₂ ·CH ₂ ·CH ₂ · 	H—	H—	175
AMYLOCAINE HYDROCHLORIDE B.P.C. ... Stovaine	—C (Me) (Et)·CH ₂ ·NMe ₂	H—	H—	178
COCAINE B.P.	MeOOC·CH—CH—CH ₂ · 	H—	H—	186*
BUTYL p-AMINO BENZOATE B.P. ... Butesin Butoform Scuroforme	—CH ₂ ·CH ₂ ·CH ₂ ·CH ₂ ·	NH ₂ —	H—	187
BENZOCAINE B.P. Anaesthesia	—CH ₂ ·CH ₂ ·	NH ₂ —	H—	195
ORTHOCAINE B.P. New Orthoform	—CH ₂ ·	HO—	NH ₂ —	225 d.
BENZAMINE HYDROCHLORIDE B.P.C. ... Betacaine HCl Betaeucaine HCl Eucaine HCl		H—	H—	268 d.

* The melting point of cocaine is variously reported in the literature to be between 182° and 202°C., the variation being attributable (Smith³) to the rate and conditions of heating.

IDENTIFICATION OF LOCAL ANÆSTHETICS



FIG. 1. Amydracaine (Dichromate)



FIG. 2. Amydracaine (Potassium Iodide)

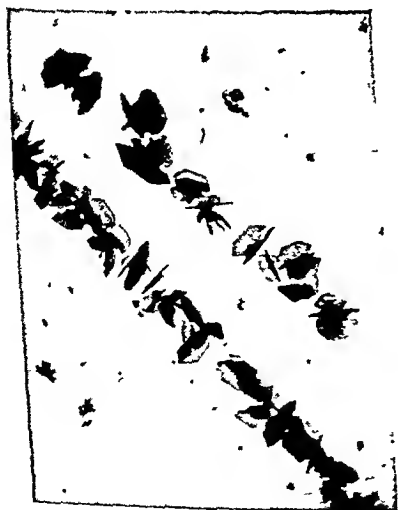


FIG. 3. Amydracaine (Permanganate).

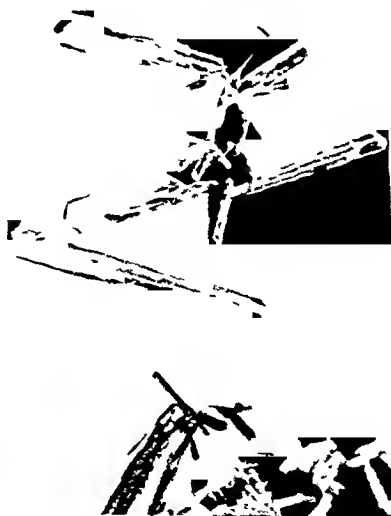


FIG. 4. Amylocaine (Potassium Iodide).

IDENTIFICATION OF LOCAL ANÆSTHETICS

property constitutes a convenient means of purification. Unfortunately, in several instances, notably with procaine, hydrolysis of the ester rapidly occurs during the process, and the initial white precipitate gradually disappears. Care must therefore be taken to avoid addition of any appreciable excess of hydrogen chloride; the following technique has been found to give the most satisfactory results.

The impure basic residue is dissolved in the smallest possible quantity of dry ether, and the solution transferred to a microcentrifuge tube. With the aid of a pipette a few drops of dry ether saturated with hydrogen chloride are carefully added. An immediate white precipitate is obtained which is usually crystalline, but which may initially be of an oily or pasty consistency. After centrifuging, the supernatant liquid is removed, and the residue is dissolved in a minimum amount of alcohol. Addition of ether to this solution causes re-precipitation of the hydrochloride either in crystalline form, or as a gummy product which readily crystallises on standing. From the melting-point of the hydrochloride, taken at this stage, an indication of the identity of the compound is usually obtained (Table I).

In view of the successful results obtained with the local anæsthetic bases, the process is being further investigated as a possible general method for purification of alkaloids and other organic compounds of basic character.

Behaviour with "alkaloidal reagents." Since all the drugs of this group are precipitated by Sonnenschein's phosphomolybdic acid reagent, this test, although in no way specific, is a useful preliminary measure, particularly in the cases of benzocaine and butyl *p*-aminobenzoate, which as indicated above, are extracted chiefly in the "acidic" group. The other common alkaloidal reagents, Mayer's potassio-mercuric iodide, and Wagner's iodine potassium iodide, also provide evidence of a confirmatory nature. In Table II the behaviour of the drugs with these three

TABLE II
REACTIONS OF LOCAL ANÆSTHETICS WITH 'ALKALOIDAL REAGENTS'

	Sonnenschein's Reagent	Mayer's Reagent	Wagner's Reagent
Amethocaine	+	+	+
Butacaine	+	+	+
Procaine	+	+	+
Amydricaine	+	+	+
Metycaine	+	+	+
Amylocaine	+	+	+
Cocaine	+	+	+
Butyl <i>p</i> -aminobenzoate	+	+	+
Benzocaine	+	+	+
Orthocaine	+	+	+
Benzamine	+	+	+

reagents is summarised. The tests are most conveniently carried out by dissolving approximately 0.1 mg. of purified hydrochloride in a drop of water, dividing this solution into three separate drops on a black tile, and applying the alkaloidal reagents one to each drop.

Demonstration of benzoic ester structure. The characteristic group

feature of these drugs is the benzoic ester structure. Although no specific test for this typical structure is available, the presence both of an ester and of an aromatic nucleus (with or without amino substituents) may readily be proved (Table III).

TABLE III
REACTIONS FOR THE BENZOIC ESTER STRUCTURE

	Hydroxamic acid reaction (ester linkage)	Alcoholysis (benzoic ester)	Soda lime/furfural reaction (aryl amino group)	Furfural reaction (free-NH ₂)
Amethocaine...	+	—	+	—
Butacaine ...	+	—	+	+
Procaine ...	+	—	+	+
Amydracaine ...	+	—	+	—
Metycaine ...	+	+	—	—
Amylocaine ...	+	+	—	—
Cocaine ...	+	+	—	—
Butyl <i>p</i> -aminobenzoate ...	+	—	—	—
Benzocaine ...	+	—	+	+
Orthocaine ...	+	—	+	+
Benzamine ...	+	+	—	—

(1) 0.1 mg. of the test material is placed in a white porcelain crucible, and to it is added 1 drop of freshly-prepared saturated alcoholic solution of hydroxylamine hydrochloride and 1 drop of freshly-prepared alcoholic potash. The crucible is gently heated until evaporation is complete, when 1 drop of N/2 hydrochloric acid and 1 drop of 1 per cent. aqueous ferric chloride solution are added. A violet-red colour indicates the presence of an ester of a carboxylic acid (Feigl, Anger and Frehden²).

(2) 0.1 mg. of material, contained in a test tube (4.5 cm. × 0.5 cm.) is treated with 3 or 4 drops of alcohol and 1 small drop of concentrated sulphuric acid. The tube is warmed over a micro-flame until most of the alcohol has evaporated. Under these conditions alcoholysis results, and the odour of ethyl benzoate is obtained with those compounds in which the aromatic nucleus is unsubstituted.

(3) 0.1 mg. of the test substance, intimately mixed with an equal weight of soda-lime, is heated in a test tube (4.5 cm. × 0.5 cm.) until white fumes are evolved, and an oily distillate approaches to within approximately 0.5 cm. from the open end of the tube. The tube is placed on a white tile, and a pointed strip of filter paper moistened with a 2 per cent. solution of furfural in glacial acetic acid is carefully introduced. A reddish-violet band around the tube in the region of the oily distillate is indicative of aniline bases. Positive reactions are obtained in this test with the amino substituted drugs.

From the results of these three tests an unknown compound may be allocated to this group of drugs with reasonable certainty, and further, within the group, the presence or absence of amino substituents in the aromatic nucleus may be verified. It is recommended that control tests be carried out with typical drugs; under these conditions, and with a little practice, it is possible to utilise even smaller quantities of test material than the 0.1 mg. specified.

One drug only amongst the listed anæsthetics, amethocaine, contains

IDENTIFICATION OF LOCAL ANÆSTHETICS

an aryl amino group which is itself substituted. Whilst, therefore, this compound, in common with the other primary amino derivatives, gives a positive reaction in Test 3 above, it differs from the other members by giving a negative response to the furfural test for a free primary amino group; 0.1 mg. of test material is treated in a white porcelain dish with 1 drop of 2 per cent. furfural in glacial acetic acid, and the liquid allowed to evaporate spontaneously, when a free $-NH_2$ group is indicated by the production of an intense red colour, rapidly turning reddish-violet (Table III).

Crystal and colour reactions. Valuable information as to the identity of a local anæsthetic drug is provided by the following three crystal reactions, which are in many instances completely diagnostic.

(1) *Potassium iodide test.* 0.01 to 0.1 mg. of test material in the form of the hydrochloride is dissolved in a drop of water on a microscope slide; if the material under examination is the free base it is dissolved in a drop of 2N hydrochloric acid. A few particles of finely ground potassium iodide are sprinkled over the surface of the drop, when a positive reaction is indicated by the immediate production of a white turbidity visible to the naked eye. On microscopic examination ($\times 50$) the appearances summarised in Table IV are observed, and for reference purposes the typical crystal forms are given in the photomicrographs.

(2) *Potassium permanganate test.* 0.1 mg. of the drug is dissolved on a microscope slide in a drop of either water or 2N hydrochloric acid as in Test (1). 1 drop of 5 per cent. alum solution is added, followed by a drop of 1 per cent. potassium permanganate solution. After stirring with a glass rod, the slide is examined microscopically ($\times 50$). In Table IV are listed the reactions of the various drugs, whilst Figures 3 and 8 show the characteristic appearances of the only two compounds, amydracaine and cocaine, which yield crystals under these conditions.

(3) *Potassium dichromate test.* 0.1 mg. of material is dissolved on a microscope slide as in the previous tests, and 1 drop of saturated potassium dichromate solution is added. The naked eye and microscopic ($\times 50$) appearances are noted; 1 drop of concentrated hydrochloric acid is then added, and the appearance again observed (Table IV). Although two compounds only, amydracaine and amethocaine, yield crystals (Figs. 1, 5) under the conditions of the test, the colour reactions of the remaining drugs are in many instances quite distinctive.

The drug orthocaine is distinguished from the other benzoic ester local anæsthetics by the presence of a phenolic $-OH$ group. When a crystal of the solid, either base or hydrochloride, is treated with a drop of an aqueous solution of ferric chloride, there is produced a dull blue colour passing to a dull green.

Mixed melting-point determination. The mixed melting-point of the hydrochloride should in all instances be the final test for identity, the

TABLE IV
CRYSTAL AND COLOUR REACTIONS

		Potassium iodide	Potassium permanganate	Potassium dichromate	
				Without concentrated hydrochloric acid	After adding concentrated hydrochloric acid
Amethocaine	...	Dense rosettes of rod-shaped crystals	Amorphous precipitate ; violet \rightarrow greenish-brown	Feathery, yellow needle-growths	Needles disappear giving brownish-green flocculent precipitate
Butacaine	...	Minute globules giving masses of fine needles	Amorphous precipitate ; violet \rightarrow greenish-brown	Yellow globules	Transient pale violet colour, then brownish-violet globules
Procaine	...	Minute globules slowly giving large hexagonal plates (crystallisation occurs more readily if a somewhat larger quantity of KI is used)	Amorphous precipitate ; violet \rightarrow greenish-brown	Yellow globules	Violet colour, then brownish-violet flocculent precipitate
Amydracaine	...	Arborescent needle growths	Violet coloured hexagonal plates	Yellow crystals in two forms : rhombs and long flat needles	Crystals dissolve
Metycaine	...	Minute globules giving large pointed needles tending to rosette formation	Amorphous precipitate ; with reddish-brown globules	Yellow globules	Yellow globules unchanged, except for slight darkening
Amylocaine	...	Thick rods, usually in aggregates	Violet solution slowly depositing brown amorphous precipitate	Yellow globules	Globules dissolve
Cocaine	...	—	Violet coloured rectangular plates	Yellow globules	Yellow flocculent precipitate
Butyl <i>p</i> -aminobenzoate		—	Amorphous brown precipitate	Yellow globules	Violet colour, then brownish-violet flocculent precipitate
Benzocaine	...	—	Amorphous brown precipitate	—	Brownish-violet flocculent precipitate gradually darkening
Orthocaine	...	—	Amorphous precipitate ; violet \rightarrow greenish-brown	Bluish-black flocculent precipitate changing to greenish-black	Precipitate gradually dissolves with development of greenish colour
Benzamine	...	Minute globules giving rosettes of long, very fine needles	—	Yellow globules	Globules gradually flocculate

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determination being conveniently carried out in a micro-apparatus of the Kofler or similar pattern.

SUMMARY

1. A scheme is described for the characterisation and identification of the eleven local anæsthetic drugs of the benzoic ester group at present commercially available in this country.

2. The complete scheme is designed in five distinct stages, any of which may be omitted if identification is otherwise satisfactorily achieved:

- (i) purification of the crude material by precipitation of the hydrochloride from an ethereal solution of the base;
- (ii) reactions with the common alkaloidal reagents;
- (iii) demonstration of the presence of the benzoic ester structure;
- (iv) simple crystal tests and colour reactions with the three reagents, potassium iodide, potassium permanganate and potassium dichromate.
- (v) final identification by micro-mixed melting-point determination.

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THE EVALUATION OF THE BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL AND SOME OF ITS MONOALKYL ETHERS AGAINST *BACTERIUM COLI*

PART VI

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PREVIOUS communications in this series^{1,2,3,4} have illustrated how the disinfection data of *Bact. coli* could be transformed into a relationship suitable for statistical treatment; this was achieved by plotting percentage survivors as probits against log. survivor time. Although it had been shown that the section of the regression between probits 4 and 6 was not strictly linear, critical analysis had indicated⁵ that there was no significant difference between the slopes of regressions obtained from different concentrations of the disinfectant (ethylene glycol) and of regressions obtained from the same concentration. It had also been argued that a disinfectant-organism reaction has a characteristic probit-log time regression which should remain constant under standard experimental conditions.

In the present paper, the regression coefficients of higher members of the homologous series (the monoalkyl ethers from methyl to hexyl) have been determined, together with the confidence limits of the estimations. Physical constants of these compounds have been published in Appendix I of Part I¹ of this series of papers. Preliminary experiments with the monoamyl ether indicated that it was so sparingly soluble in water that even the saturated solution showed very little bactericidal activity. Investigations with this compound were therefore discontinued.

DISINFECTION STUDIES IN THE ETHYLENE GLYCOL MONOALKYL ETHERS AT 20°C.

Probit-log time regressions.

Experimental part.—Concentrations of the ethers were prepared and their disinfectant activities tested against *Bact. coli* by means of the standardised technique¹. In most instances four tests were carried out at each concentration.

Results and calculations.—Probit-log. time regressions were calculated for each test. Summaries of the terms necessary to calculate the mean slopes and for the calculation of the error mean square at each concentration are submitted in Tables IA to VD. Those for ethylene glycol are to be found in Part V⁵ of this series of papers.

Combined data from the calculations of the probit-log time regressions for ethylene glycol and its monoalkyl ethers at 20°C.

Table VI presents a summary of the massed statistical data from the calculations of probit-log. time regressions for concentrations of ethylene glycol and its monoalkyl ethers at 20°C. From it has been calculated

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the mean slope ($\bar{b}=1.2040$) and the sum of squares for the joint regression (224.508804).

The analysis of variance of the massed regressions is presented in Table VII. The mean square for the residual in y (0.044293) has been used as denominator to calculate the variance ratios between the variation in regression between compounds, variation in regression between concentrations, and variation in regression between tests; these have been included in Table VIII. Owing to the large number of degrees of freedom

TABLE IA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 20°C.

Observation	Concentrations of ethylene glycol monomethyl ether				
	42.5 per cent.	45.0 per cent.	47.5 per cent.	50.0 per cent.	52.5 per cent.
$S[(x-\bar{x})(y-\bar{y})]$	1.994529	3.189758	4.013346	3.873828	2.923893
$S(x-\bar{x})^2$	4.170328	4.148876	5.797243	4.490993	2.307026
$S(y-\bar{y})^2$	1.485401	3.219608	4.575887	3.857329	3.972279
N	11	11	13	13	9
SS for individual regressions ...	1.075110	2.522587	3.167978	3.585350	3.763250
b	0.478267	0.768825	0.692285	0.862577	1.267386
SS pool	0.953917	2.452364	2.778380	3.341476	3.705702

TABLE Ib

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 20°C.

Concentration	Item	Sum of squares	N	Mean square
42.5 per cent.	Common regression ...	0.953917	1	0.953917
	Variation in regression ...	0.121183	3	0.040394
	Total	1.075100	4	
	Residual in y	0.410291	11	0.037299
45.0 per cent.	Common regression ...	0.452354	1	0.452354
	Variation in regression ...	0.070323	3	0.023441
	Total	2.522687	4	
	Residual in y	0.696921	11	0.063356
47.5 per cent.	Common regression ...	2.778380	1	2.778380
	Variation in regression ...	0.389598	3	0.129866
	Total	3.167978	4	
	Residual in y	1.407909	13	0.108301
50.0 per cent.	Common regression ...	3.341476	1	3.341476
	Variation in regression ...	0.243874	3	0.081291
	Total	3.585350	4	
	Residual in y	0.273979	13	0.021075
52.5 per cent.	Common regression ...	3.705702	1	3.705702
	Variation in regression ...	0.057548	3	0.019183
	Total	3.763250	4	
	Residual in y	0.209029	9	0.023225

TABLE Ic

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 20°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
42.5 per cent. ...	0.410291	11	0.121183	3	0.953917	1.994529	4.170328
45.0 " " ...	0.696921	11	0.070323	3	2.452364	3.189758	4.148876
47.5 " " ...	1.407909	13	0.389598	3	2.778380	4.013346	5.797243
50.0 " " ...	0.273979	13	0.243874	3	3.341476	3.873838	4.490993
42.5 " " ...	0.209029	9	0.057548	3	3.705702	2.923893	2.307026
Totals ...	2.998129	57	0.882526	15	13.231839	15.995354	20.914466

$$\bar{b} = \frac{15.995354}{20.914466} = 0.764799$$

$$SS \text{ for joint regression} = \frac{(15.995354)^2}{20.914466} = 12.233224$$

TABLE Id

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 20°C.

Item	N	Sum of squares	Mean square
Grand regression ...	1	12.233224	12.233224
Variation in regression between concentrations ...	4	0.998615	0.249654
Variation in regression within concentrations ...	15	0.882526	0.058835
Residual in y ...	57	2.998129	0.052599

TABLE IIA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 20°C.

Observation	Concentrations of ethylene glycol monoethyl ether				
	25.0 per cent.	27.5 per cent.	30.0 per cent.	32.5 per cent.	35.0 per cent.
$S[(x-\bar{x})(y-\bar{y})]$...	2.273209	1.570023	5.630061	3.867540	2.473940
$S(x-\bar{x})^2$...	2.325423	2.284708	6.834377	3.203024	2.463525
$S(y-\bar{y})^2$...	2.348405	1.286192	5.179256	5.533561	2.907313
N ...	8	7	15	11	11
SS for individual regressions ...	2.233417	1.200121	4.931650	5.002978	2.549408
b ...	0.977547	0.687188	0.823786	1.207465	1.004228
SS pool ...	2.223167	1.078900	4.637963	4.669920	2.484399

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involved it is not possible to use the ordinary statistical tables in order to compute the probabilities; this difficulty was overcome by calculating z as exemplified by Mather⁶ (section 16). The expected values of z for the variance ratios have been calculated from the relationship $z = \frac{1}{2} \log$. (Variance Ratio); these have been set out in Table VIII. The calculated values of z have been compared with the theoretical values, to give the

TABLE IIb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 20°C.

Concentration	Item	Sum of squares	N	Mean square
25.0 per cent.	Common regression ...	2.222167	1	2.222167
	Variation in regression ...	0.011250	2	0.005625
	Total	2.011250	3	
	Residual in y ...	0.114988	8	0.014373
27.5 per cent.	Common regression ...	1.078900	1	1.078900
	Variation in regression ...	0.121221	1	0.121221
	Total	1.200121	2	
	Residual in y ...	0.086071	7	0.012296
30.0 per cent.	Common regression ...	4.637963	1	4.637963
	Variation in regression ...	0.293687	3	0.097896
	Total	4.931650	4	
	Residual in y ...	0.247606	15	0.016507
32.5 per cent.	Common regression ...	4.669920	1	4.669920
	Variation in regression ...	0.333058	4	0.084265
	Total	5.002978	5	
	Residual in y ...	0.526583	00	0.047871
35.0 per cent.	Common regression ...	2.484399	1	2.484399
	Variation in regression ...	0.065009	3	0.021669
	Total	2.549408	4	
	Residual in y ...	0.357905	11	0.032537

TABLE IIc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 20°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
25.0 per cent. ..	0.111498	8	0.011250	2	2.222167	2.273209	2.325423
27.5 " " . . .	0.086071	7	0.121221	1	1.078900	1.570023	2.284708
30.0 " " . . .	0.247606	15	0.293687	3	4.637963	5.630061	6.834377
32.5 " " . . .	0.526583	11	0.333058	4	4.669920	3.867540	3.203024
35.0 " " . . .	0.357905	11	0.065009	3	2.484399	2.473940	2.463525
Totals . . .	1.333153	52	0.824225	13	15.093349	15.814773	17.111057

$$\bar{b} = \frac{15.814773}{17.111057} = 0.924243$$

$$SS \text{ for joint regression} = \frac{(15.814773)^2}{17.111057} = 14.616692$$

probabilities of the significance between the items selected; these have also been included in Table VIII.

Test of significance of the difference between the mean squares for the variation in regression between concentrations and variation in regression between individual tests.

The formula for high values of N_1 and N_2 is given in Table V (Distribution of z) of the Statistical Tables of Fisher and Yates⁷.

$$z_{(5 \text{ per cent})} = \frac{1.6499}{\sqrt{h-1}} - 0.7843 \left(\frac{1}{N_1} - \frac{1}{N_2} \right) \text{ approximately,}$$

$$\text{where } \frac{2}{h} = \frac{1}{N_1} + \frac{1}{N_2}$$

$$N_1 = 27 \text{ and } N_2 = 172, \text{ therefore } h = 46.6734.$$

$$\text{Hence } z_{(5 \text{ per cent})} = \frac{1.6449}{\sqrt{46.6734 - 1}} - (0.7843 \times 0.0132) = 0.2189$$

The observed value of z (0.082202) is less than that calculated at the 5 per cent. level, hence $P > 0.05$ and the difference is not significant.

TABLE IIb

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG TIME REGRESSIONS FROM DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 20°C.

Item	<i>N</i>	Sum of squares	Mean square
Grand regression	1	14 616692	14 616692
Variation in regression between concentrations	4	0 476657	0 119164
Variation in regression within concentrations	13	0 824325	0 062948
Residual in <i>y</i>	52	1 333153	0 025638

TABLE IIIa

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS OF DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 20°C.

Observation	Concentrations of ethylene glycol monopropyl ether				
	7 8 per cent	9 0 per cent	10 0 per cent	11 0 per cent	12 0 per cent
$S[(x-\bar{x})(y-\bar{y})]$	4 589090	2 749305	3 868675	2 837293	1 771908
$S(x-\bar{x})^2$	2 620754	1 649256	1 970901	1 710080	0 847386
$S(y-\bar{y})^2$	9 647604	5 729806	8 440537	5 643021	5 642021
<i>N</i>	12	7	10	9	4
SS for individual regressions	8 184688	5 574039	7 776921	4 772751	4 732316
<i>b</i>	1 751057	1 666997	1 962897	1 659158	2 093383
SS pool	8 035759	4 583084	7 593509	4 707518	3 713478

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The two errors may therefore be pooled (as in Table VII); the combined sum of squares is 19.633745 for 199 degrees of freedom, with a mean square of 0.098662. This figure therefore represents the error mean square of the estimation of the slopes of all the regressions for ethylene glycol and its monoalkyl ethers at 20°C., and is used later in determining the standard errors.

TABLE IIIb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 20°C.

Concentration	Item	Sum of squares	N	Mean square
7.8 per cent.	Common regression ...	8.035759	1	8.035759
	Variation in regression ...	0.148929	3	0.049643
	Total	8.184688	4	0.121909
	Residual in y	1.462916	12	
9.0 per cent.	Common regression ...	4.583084	1	4.583084
	Variation in regression ...	0.990955	3	0.330318
	Total	5.574039	4	0.022252
	Residual in y	0.155767	7	
10.0 per cent.	Common regression ...	7.593809	1	7.593.809
	Variation in regression ...	0.183112	3	0.061037
	Total	7.776921	4	0.007699
	Residual in y	0.060270	9	
11.0 per cent.	Common regression ...	4.707518	1	4.707518
	Variation in regression ...	0.065233	3	0.021744
	Total	4.772751	4	0.007699
	Residual in y	0.069270	9	
12.0 per cent.	Common regression ...	3.713478	1	3.713478
	Variation in regression ...	1.018838	3	0.272946
	Total	4.732316	4	0.277426
	Residual in y	1.109705	4	

TABLE IIIc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 20°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
7.8 per cent. ...	1.462916	12	0.148929	3	8.035759	4.589090	2.620754
9.0 " " ...	0.155767	7	0.990955	3	4.583084	2.749305	1.649256
10.0 " " ...	0.663616	10	0.183112	3	7.593809	3.868675	1.970901
11.0 " " ...	0.069270	9	0.065233	3	4.707518	2.837293	1.710080
12.0 " " ...	1.109705	4	1.018838	3	3.713478	1.773908	0.847386
Totals	3.461274	42	2.407067	15	28.633648	15.818271	8.798377

$$\bar{b} = \frac{15.818271}{8.798377} = 1.797864$$

$$SS \text{ for joint regression} = \frac{(15.818271)^2}{8.798377} = 28.439074$$

Test of significance of the difference between the mean squares for the variation in regression between the different compounds and the residual in y .

Here $N_1 = 5$ and $N_2 = 557$; P may be found by direct consultation of the table showing the distribution of z . The observed value (0.994715) is larger than the theoretical value even at the 0.1 per cent. level (which is about 0.7), hence $P < 0.001$.

Test of significance of the difference between the mean squares for the variation in regression between concentrations and the residual in y .

Here $N_1 = 27$ and $N_2 = 557$, therefore $h = 51.5034$; from this, z (5 per cent.) = 0.20376.

The observed value of z (0.268825) is slightly greater than that calculated at the 5 per cent. level and hence $P < 0.05$.

$$z \text{ (1 per cent.)} = \frac{2.3263}{\sqrt{h-1.4}} - 1.235 \left(\frac{1}{N_1} - \frac{1}{N_2} \right) \text{ approximately,}$$

$$= \frac{2.3263}{\sqrt{51.5034 - 1}} - 1.235 (0.03524) = 0.2852.$$

TABLE IIIb

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 20°C.

Item	N	Sum of squares	Mean square
Grand regression	1	28.439074	28.439074
Variation in regression between concentrations ...	4	0.194574	0.048644
Variation in regression within concentrations ...	15	2.407067	0.160471
Residual in y	42	3.461274	0.082411

TABLE IVA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 20°C.

Observation	Concentrations of ethylene glycol monobutyl ether				
	3.5 per cent.	3.75 per cent.	4.0 per cent.	4.25 per cent.	4.5 per cent.
$S[(x-\bar{x})(y-\bar{y})]$	4.059036	2.358281	3.279052	3.267227	2.335002
$S(x-\bar{x})^2$	2.525145	1.856849	2.038721	2.656913	1.714294
$S(y-\bar{y})^2$	7.013784	5.127533	5.590826	5.812414	3.560732
N	10	6	10	13	7
SS for individual regressions ...	6.696465	4.799289	5.470142	4.155352	3.443844
b	1.607447	1.270045	1.608387	1.229708	1.362078
SS pool	6.524684	2.995122	5.273984	4.017735	3.180455

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The observed value of z is smaller than that calculated at the 1 per cent. level; hence $P \approx 0.05$ to 0.01 .

Test of significance of the difference between the mean squares for the variation in regression between tests and the residual in y .

$$z_{(0.1 \text{ per cent})} = \frac{3.0902}{\sqrt{h-2.1}} - 1.925 \left(\frac{1}{N_1} - \frac{1}{N_2} \right) \text{ approximately.}$$

Here, $N_1 = 172$ and $N_2 = 557$, therefore $h = 262.837$.

$$\text{Hence } z_{(0.1 \text{ per cent})} = \frac{3.0902}{\sqrt{262.837 - 2.1}} - 1.925 (0.004) = 0.1838$$

The observed value of z (0.186797) is greater than that calculated at the 0.1 per cent. level, hence $P < 0.001$.

Test of significance of the difference between the mean squares for the variation in regression between the pooled error (i.e. variation between concentrations + between tests) and the residual in y .

Here $N_1 = 199$ and $N_2 = 557$, hence $h = 292.24$.

$$\text{Hence } z_{(0.1 \text{ per cent})} = \frac{3.0902}{\sqrt{292.24 - 2.1}} - 1.925 (0.0032) = 0.1749$$

The observed value of z (0.20021) is greater than that calculated at the 0.1 per cent. level, hence $P < 0.001$.

TABLE IVB

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 20°C .

Concentration	Item	Sum of squares	N	Mean square
3.50 per cent	Common regression	6 524684	1	6 524684
	Variation in regression	0 071781	3	0 057260
	Total Residual in y	6 696465 0 317319	4 10	0 031732
3.75 per cent	Common regression	2 995122	1	2 995122
	Variation in regression	1 804167	3	0 601389
	Total Residual in y	4 799289 0 328244	4 6	0 054707
4.00 per cent	Common regression	5 273984	1	5 273984
	Variation in regression	0 196158	3	0 653860
	Total Residual in y	5 470142 0 120684	4 10	0 012068
4.25 per cent	Common regression	4 017735	1	4 017735
	Variation in regression	0 137617	3	0 045872
	Total Residual in y	4 155352 1 657062	4 13	0 127466
4.50 per cent	Common regression	3 180455	1	3.180455
	Variation in regression	0 263389	3	0 087796
	Total Residual in y	3 443844 0 116888	4 7	0 016697

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TABLE IVc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 20°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
3.50 per cent. ...	0.317319	10	0.171781	3	6.524684	4.059036	2.525145
3.75 " " ...	0.328244	6	1.804167	3	2.995122	2.358281	1.856849
4.00 " " ...	0.120684	10	0.196158	3	5.273984	3.279052	2.038721
4.25 " " ...	1.657062	13	0.137617	3	4.017735	3.267227	2.656913
4.50 " " ...	0.116888	7	0.263389	3	3.180455	2.335002	1.714294
Totals ...	2.540197	46	2.573112	15	21.991980	15.298598	10.791922

$$\bar{b} = \frac{15.298598}{10.791922} = 1.417597$$

$$SS \text{ for joint regression} = \frac{(15.298598)^2}{10.791922} = 21.687249$$

TABLE IVd

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 20°C.

Item	N	Sum of squares	Mean square
Grand regression ...	1	21.687249	21.687249
Variation in regression between concentrations ...	4	0.304731	0.076183
Variation in regression within concentrations ...	15	2.573112	0.171541
Residual in y ...	46	2.540197	0.055222

TABLE VA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 20°C.

Observation	Concentrations of ethylene glycol monohexyl ether					
	0.400 per cent.	0.425 per cent.	0.450 per cent.	0.475 per cent.	0.500 per cent.	Miscellaneous
$S[(x-\bar{x})(y-\bar{y})]$...	6.410363	4.731915	2.950592	4.051499	4.047545	2.493439
$S(x-\bar{x})^2$...	3.410920	2.432214	1.872617	2.945145	2.889779	1.494108
$S(y-\bar{y})^2$...	12.666664	10.214115	4.703732	5.951287	6.096003	4.714120
N ...	17	12	6	10	15	15
SS for individual regressions ...	12.099071	9.629824	4.659579	5.735731	5.866990	4.496297
b ...	1.879365	1.945518	1.575652	1.375653	1.400642	1.668848
SS pool ...	12.047411	9.206024	4.649105	5.573459	5.669160	4.161170

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TABLE Vb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 20°C.

Concentration	Item	Sum of squares	N	Mean square
0.400 per cent.	Common regression ...	12.047411	1	12.047411
	Variation in regression ...	0.051660	4	0.012915
	Total	12.099071	5	
	Residual in y	0.567593	17	0.033388
0.425 per cent.	Common regression ...	9.206024	1	9.206024
	Variation in regression ...	0.423800	4	0.105950
	Total	9.629824	5	
	Residual in y	0.584291	12	0.048524
0.450 per cent.	Common regression ...	4.649105	1	4.649105
	Variation in regression ...	0.010474	1	0.010474
	Total	4.659579	2	
	Residual in y	0.044153	6	0.007356
0.475 per cent.	Common regression ..	5.573459	1	5.573459
	Variation in regression ...	0.162272	3	0.054091
	Total	5.735731	4	
	Residual in y	0.215556	10	0.021556
0.500 per cent.	Common regression ..	5.669160	1	5.669160
	Variation in regression ...	0.197830	4	0.049458
	Total	5.866990	5	
	Residual in y	0.229013	15	0.015268
Miscellaneous	Common regression ..	4.161170	1	4.161170
	Variation in regression ..	0.335127	5	0.067025
	Total	4.496297	6	
	Residual in y	0.217823	13	0.016756

TABLE Vc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 20°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
0.400 per cent.	0.567593	17	0.051660	4	12.047411	6.410363	3.410920
0.425 " "	0.584291	12	0.423800	4	9.206024	4.731915	2.432214
0.450 " "	0.044153	6	0.010474	1	4.649105	2.950592	1.872617
0.475 " "	0.215556	10	0.162272	3	5.573459	4.051449	2.945145
0.500 " "	0.229013	15	0.197830	4	5.669160	4.047545	2.889779
Miscellaneous ..	0.217823	13	0.335127	5	4.161170	2.493439	1.494108
Totals . . .	1.858429	73	1.181163	21	41.306329	24.685353	15.044783

$$\bar{b} = \frac{24.685353}{15.044783} = 1.640792$$

$$SS \text{ for joint regression} = \frac{(24.685353)^2}{15.044783} = 40.503519$$

TABLE Vd

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG TIME REGRESSIONS FROM DISINFECTATION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 20°C.

Item	N	Sum of squares	Mean square
Grand regression	1	40.503519	40.503519
Variation in regression between concentrations ...	5	0.802810	0.160562
Variation in regression within concentrations ...	21	1.181163	0.056246
Residual in y	73	1.858429	0.025458

INFERENCES FROM THE ANALYSIS

The analysis of variance indicates that there is a significant large variation in the regressions between the different compounds, i.e. each substance has its characteristic regression coefficient which differs significantly from the average of the series. The analysis also shows that the variations in the regressions between the concentrations of the substances are of the same order as the variation between the individual tests at a particular concentration. Hence the regressions of different concentrations of the same substance may be taken as parallel.

Calculation of the standard errors of the probit-log time regression coefficients at 20°C.

The variance of b is given by the formula $V_b = \frac{V_y}{S(\bar{x}-x)^2}$ (Mather⁶, section 32). The standard error, s_b , of the regression coefficient is $\sqrt{V_b}$. V_y is the error mean square from the pooled error of the variation in regression between concentrations and tests, and is 0.098662 (Table VII). The standard errors of the mean regression coefficients of all the compounds for experiments conducted at 20°C. have been computed and set out in Table IX. The ratio of the regression coefficients to their standard errors in all cases is seen to be large thereby indicating that b has been estimated satisfactorily.

Calculation of the confidence limits of the probit-log time regressions.

It is useful to present graphically the limits of error of the regression lines at a fixed probability level, ($P = 0.05$ has been chosen), so that the advantages of an increased number of experiments from which to calculate the mean, can be appreciated more readily.

(i) The first stage in this calculation is to determine the sum of the squares for the deviations of \bar{y} (the mean value of the probits in an experiment) from $\bar{\bar{y}}$ (the mean value of \bar{y} for the several tests on the same concentration of the disinfectant). It is necessary to compute this figure for every concentration used for all the compounds. Table X shows the calculations for the monomethyl ether; the calculations for the remaining compounds are precisely the same. The final figures for all compounds are included in Table XI.

(ii) The next stage is to calculate the empirical variance of \bar{y} (the mean of the mean probits). The sum of the squares of the deviations of y is divided by N (the number of degrees of freedom, i.e. one less than the number of experiments), to give the mean square. Thus in the case of 42.5

TABLE VI
SUMMARY OF MASSED STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG TIME REGRESSIONS FOR CONCENTRATIONS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 20°C.

Compound	Range of Concentrations Investigated	Residual in y		Variation in b				Grand Regression Pooled SS	$S((x-\bar{x})(y-\bar{y}))$	$S(x-\bar{x})^2$
		SS	N	Between Tests	SS	N	Between Concentrations			
Ethylene glycol	72.5 to 90.0 per cent.	12.479859	287	8.210628	93	0.727637	6	118.868425	98.850144	82.703083
Monomethyl ether	42.5 to 52.5 "	2.998129	57	0.882526	15	0.998615	4	12.233224	15.995354	20.914466
Monomethyl ether	25.0 to 35.0 "	1.333153	52	0.824225	13	0.476657	4	14.616692	15.814773	17.111057
Monopropyl ether	7.8 to 12.0 "	3.461274	42	2.407067	15	0.194574	4	28.439074	15.818171	8.798377
Monobutyl ether	3.5 to 4.5 "	2.540197	46	2.573112	15	0.104731	4	21.687249	15.298598	10.791922
Monohexyl ether	0.4 to 0.5 "	1.858429	73	1.181163	21	0.802810	5	40.503519	24.685353	15.044783
Totals	...	24.671041	557	16.078721	172	3.505024	27	236.348183	186.462493	154.863688

SS for joint regression = $\frac{(186.462493)^2}{154.863688} = 224.508864$

$b = \frac{186.462493}{154.863688} = 1.204043$

per cent. monomethyl ether (Table XI) the mean square will be $\frac{0.066713}{3} = 0.022238$.

The grand total of all the sum of squares of the deviations is divided by the total number of degrees of freedom to give the general or pooled mean square. Table XI sets out these figures from which it is seen that the average mean square is $\frac{6.43660}{165} = 0.039010$.

The variance of \bar{y} at a particular concentration is obtained by dividing the general mean square (0.039010) by the number of experiments performed at that concentration; in the instance cited above it will be $\frac{0.022238}{4}$, i.e., 0.009753.

Hence the more tests performed at a particular concentration the smaller will be the value of $V_{\bar{y}}$; thus at 75 per cent. ethylene glycol 32 experiments were used to calculate the mean and here $V_{\bar{y}} = 0.001219$, whereas at 27.5 per cent. monomethyl ether only 2 experiments were performed and its $V_{\bar{y}} = 0.019505$.

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The confidence limits will therefore vary with the number of tests performed for a particular concentration. Figure 1 has been constructed to indicate the confidence limits which are to be expected at a probability level of $P = 0.05$ when 32 tests are used to calculate the mean (as for

TABLE VII

ANALYSIS OF VARIANCE OF MASSED REGRESSIONS FOR DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 20°C.

Item	N	SS	Mean square	Variance Ratio	Probability
Massed regression	1	224.508804	224.508804	(See Table VIII)	
Variation in regression between compounds	5	11.839379	2.367876		
Variation in regression between concentrations	27	3.505024	0.129816		
Variation in regression between tests	172	16.078721	0.093481		
Residual in y	557	24.671041	0.044293		
Pooled error	199	19.633745	0.098662		

TABLE VIII

CALCULATION OF THE z 'S FOR THE ITEMS IN THE ANALYSIS OF VARIANCE OF THE MASSED REGRESSIONS IN TABLE VII

N_1/N_2	Variance ratio (V.R.)	$\log_{10} V.R.$	$\log_e V.R. = \log_{10} V.R. \times 1.15129$	$z = \frac{1}{2} \log_e V.R.$	Probability
27/172	1.388689	0.1428	0.16440	0.08220	<0.05
5/557	53.459373	1.7280	1.98943	0.99472	<0.001
27/557	2.930847	0.4670	0.53765	0.26883	0.05 to 0.01
172/557	2.110514	0.3245	0.37359	0.18680	<0.001
199/557	2.227485	0.3478	0.40042	0.20021	<0.001

TABLE IX

THE PROBIT-LOG TIME REGRESSION COEFFICIENTS WITH THEIR STANDARD ERRORS, OF THE REACTION BETWEEN *BACT. COLI* AND ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 20°C.

Compound	b	N	$S(x-\bar{x})^2$	V_y	$s_b = \frac{V_y}{S(x-\bar{x})^2}$	Ratio of b to s_b
Ethylene glycol	1.202511	93	82.203083	0.098662	0.03465	35
Monomethyl ether	0.764799	15	20.914466		0.06871	11
Monoethyl ether	0.924243	13	17.111057		0.07595	12
Monoisopropyl ether	1.797864	15	8.798377		0.10570	17
Mobobutyl ether	1.417597	15	10.791922		0.09563	15
Monobexyl ether	1.640792	21	15.044783		0.08098	20

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75 per cent. ethylene glycol), and when only 4 tests are used (as for 0.425 per cent. monohexyl ether). The calculations involved are as follows:—

(a) *Confidence limits for 32 experiments with 75 per cent ethylene glycol at 20°C.*

Confidence limits at $P = 0.05 = \pm s_y \times 1.96$ (where 1.96 = the value of the normal deviate at $P = 0.05$).

$$\text{Now } s_y = \sqrt{V_y} \text{ where } V_y = V_{\text{mean}} + V_b (x - \bar{x})^2$$

But V_y = variance of y

and V_{mean} = variance of \bar{y} (i.e. $V_{\bar{y}}$ in Table II)
 $= 0.001219$,

$$\text{and } V_b = \frac{V}{S(x - \bar{x})^2}$$

where V = error mean square = 0.098662 (Table VII)

and $S(x - \bar{x})^2 = 32.035396$ (Table VI, Part V^b)

$$\text{Hence } V_b = \frac{0.098662}{32.035396} = 0.003080$$

x = abscissa at which value of V_y is to be determined.

\bar{x} = mean value of x (i.e. log. time), in the 32 experiments.

$$= \frac{67.608}{32} = 2.115.$$

The values of V_y at values of x are obtained by substituting in the equation $V_y = V_{\text{mean}} + V_b (x - \bar{x})^2$.

For example, at $x = 1$, $V_y = 0.001219 + 0.003080 (1 - 2.115)^2$
 $= 0.005048$

$$s_y = \sqrt{0.005048} = 0.07105$$

TABLE X

CALCULATION OF THE SUM OF SQUARES FOR DEVIATIONS OF THE MEAN PROBIT (\bar{y}), FOR CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 20°C.

	Concentrations of ethylene glycol monomethyl ether									
	42.5 per cent.		45.0 per cent.		47.5 per cent.		50.0 per cent.		52.5 per cent.	
	Expt. No.	Mean Probit \bar{y}	Expt. No.	Mean Probit \bar{y}	Expt. No.	Mean Probit \bar{y}	Expt. No.	Mean Probit \bar{y}	Expt. No.	Mean Probit \bar{y}
	208a 209f 210c 211c	4.414 4.503 4.759 4.617	208d 209e 210d 211d	4.720 4.516 5.064 5.119	209c 211e 213e 214c	4.797 4.891 5.126 5.070	209d 210f 211f 212d	5.092 5.591 5.423 4.807	210g 211g 212e 213d	5.853 5.870 5.169 5.183
No of expts. $\frac{S(\bar{y})}{\bar{y}}$	18-293 4 4.573		19-419 4 4.855		19-884 4 4.971		20-913 4 5.228		22-075 4 5.519	
$\frac{S(\bar{y})^2}{S^2(\bar{y})}$	83.725175		94.520913		98.913866		109.703923		122.296559	
$\frac{S(\bar{y})^2}{n}$	83.658462		94.274390		98.843364		109.338392		121.826406	
$\frac{S(\bar{y} - \bar{\bar{y}})^2}{n}$ $= \frac{S(\bar{y})^2}{n} - \frac{S^2(\bar{y})}{n}$	0.066713		0.246523		0.070532		0.365531		0.470153	
= SS										

Confidence limits (at $P = 0.05$) = $0.07105 \times 1.96 = \pm 0.1396$ probits.
 Confidence limits (at $P = 0.05$) have been calculated for a number of values of x and the results are set out in Table XII.

TABLE XI

THE EMPIRICAL VARIANCE OF THE INDIVIDUAL MEANS FROM THEIR MEAN PROBIT OF EXPERIMENTS WITH CONCENTRATIONS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 20°C.

Compound	Concentration per cent	SS	N	Mean square	$V_{\bar{y}}$
Ethylene glycol	72.5	0.318245	11	0.028931	0.003251
	75.0	0.476395	31	0.015368	0.001219
	77.5	0.411183	14	0.029370	0.002601
	80.0	0.406955	9	0.045217	0.003901
	82.5	0.307976	8	0.038497	0.004334
	85.0	0.238682	9	0.026520	0.003901
	90.0	0.617642	9	0.068627	0.003901
Monomethyl ether	42.5	0.066713	3	0.022238	0.009753
	45.0	0.246523	3	0.082174	0.009753
	47.5	0.070532	3	0.023511	0.009753
	50.0	0.365531	3	0.121877	0.009753
	52.5	0.470513	3	0.156838	0.009753
Monoethyl ether	25.0	0.141523	2	0.070762	0.013003
	27.5	0.000420	1	0.000420	0.019505
	30.0	0.086265	3	0.028755	0.009753
	32.5	0.316301	4	0.079075	0.007802
	35.0	0.133905	3	0.044635	0.009753
Monopropyl ether	7.8	0.110310	3	0.036770	0.009753
	9.0	0.174145	3	0.058048	0.009753
	10.0	0.076907	3	0.025636	0.009753
	11.0	0.051941	3	0.017314	0.009753
	12.0	0.120228	3	0.040076	0.009753
Monobutyl ether	3.50	0.216277	3	0.072092	0.009753
	3.75	0.411050	3	0.137017	0.009753
	4.00	0.146367	3	0.048789	0.009753
	4.25	0.033001	3	0.011000	0.009753
	4.50	0.078645	3	0.026215	0.009753
Monohexyl ether	0.375	0.000061	1	0.000061	0.019503
	0.400	0.038307	4	0.009577	0.007802
	0.425	0.089235	3	0.029745	0.009753
	0.450	0.016928	1	0.016928	0.019503
	0.475	0.149173	3	0.049724	0.009753
	0.500	0.048775	4	0.012194	0.007802
Totals	—	6.436660	165	0.039010	—

TABLE XII

CONFIDENCE LIMITS AT $P = 0.05$ FOR VALUES OF x FOR 32 EXPERIMENTS WITH 75 PER CENT. ETHYLENE GLYCOL, AND 4 EXPERIMENTS WITH 0.425 PER CENT. MONOHEXYL ETHER, AT 20°C.

Value of x	Confidence Limits for 32 experiments (75 per cent ethylene glycol)	Confidence Limits for 4 experiments (0.425 per cent monohexyl ether)
1.0	± 0.1393 probits	± 0.4214 probits
1.2	0.1208 "	0.3130 "
1.4	0.1017 "	0.2903 "
1.6	0.0884 "	0.2376 "
1.8	0.0765 "	0.2021 "
2.0	0.0696 "	0.1946 "
2.2	0.0690 "	0.2178 "
2.4	0.0727 "	0.2632 "
2.6	0.0864 "	0.3191 "
2.8	0.1012 "	0.3848 "

(b) Confidence limits for 4 experiments with 0.425 per cent. monohexyl ether at 20°C.

$$V_b = \frac{V}{S(x-\bar{x})^2} \text{ where } V = 0.098662 \text{ (Table VII.)}$$

$$\text{and } S(x-\bar{x})^2 = 2.432214 \text{ (Table Va.)}$$

$$= \frac{0.098662}{2.432214} = 0.040565$$

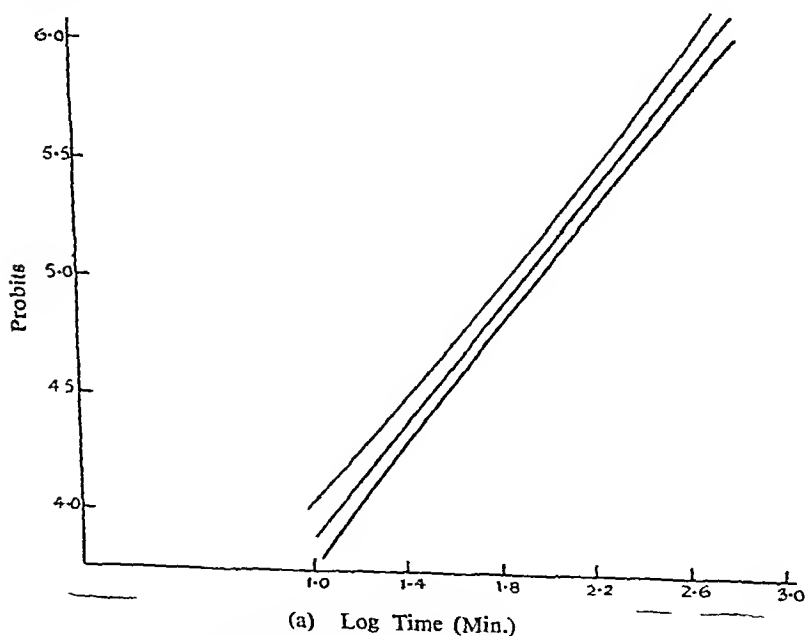
$$S(\bar{x}) = 7.791, \text{ therefore } \bar{\bar{x}} = \frac{7.791}{4} = 1.948$$

The values of V_y at values of x are obtained by substituting in the equation $V_y = V_{mean} + V_b(x-\bar{x})^2$, where $V_{mean} = V_{\bar{y}} = 0.009753$ (Table XI.)

For example: at $x = 1$, $V_y = 0.009753 + 0.040565(1000 + 1.948)^2 = 0.046209$; $s_y = \sqrt{0.046209} = \pm 0.215$.

Confidence limits (at $P=0.05$) have been calculated for a number of values of x . The results are set out in Table XII.

Figure 1 has been constructed (from the results in Table XII) to show graphically the limits of error (a) when 32 experiments are used to compute the mean and (b) when 4 experiments are used.



—Confidence limits (at $P = 0.05$) of probit-log time regressions
 1 32 expts. of 75 per cent. ethylene glycol at 20°C.

Confidence limits (at $P = 0.05$) = $0.07105 \times 1.96 = \pm 0.1396$ probits.
 Confidence limits (at $P = 0.05$) have been calculated for a number of values of x and the results are set out in Table XII.

TABLE XI

THE EMPIRICAL VARIANCE OF THE INDIVIDUAL MEANS FROM THEIR MEAN PROBIT OF EXPERIMENTS WITH CONCENTRATIONS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 20°C.

Compound	Concentration per cent	SS	N	Mean square	$V_{\bar{y}}$
Ethylene glycol	72.5	0.318245	11	0.028931	0.003251
	75.0	0.476395	31	0.015368	0.001219
	77.5	0.411183	14	0.029370	0.002601
	80.0	0.406955	9	0.045217	0.003901
	82.5	0.307976	8	0.038497	0.004334
	85.0	0.238682	9	0.026520	0.003901
	90.0	0.617642	9	0.068627	0.003901
Monomethyl ether	42.5	0.066713	3	0.022238	0.009753
	45.0	0.246523	3	0.082174	0.009753
	47.5	0.070532	3	0.023511	0.009753
	50.0	0.365531	3	0.121877	0.009753
	52.5	0.470513	3	0.156838	0.009753
Monoethyl ether	25.0	0.141523	2	0.070762	0.013003
	27.5	0.000420	1	0.000420	0.019505
	30.0	0.086265	3	0.028755	0.009753
	32.5	0.316301	4	0.079075	0.007802
	35.0	0.133905	3	0.044635	0.009753
Monopropyl ether	7.8	0.110310	3	0.036770	0.009753
	9.0	0.174145	3	0.058048	0.009753
	10.0	0.076907	3	0.025636	0.009753
	11.0	0.051941	3	0.017314	0.009735
	12.0	0.120228	3	0.040076	0.009753
Monobutyl ether	3.50	0.216277	3	0.072092	0.009753
	3.75	0.411050	3	0.137017	0.009753
	4.00	0.146367	3	0.048789	0.009753
	4.25	0.033001	3	0.011000	0.009753
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BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL—PART VI

(b) Confidence limits for 4 experiments with 0.425 per cent. monohexyl ether at 20°C.

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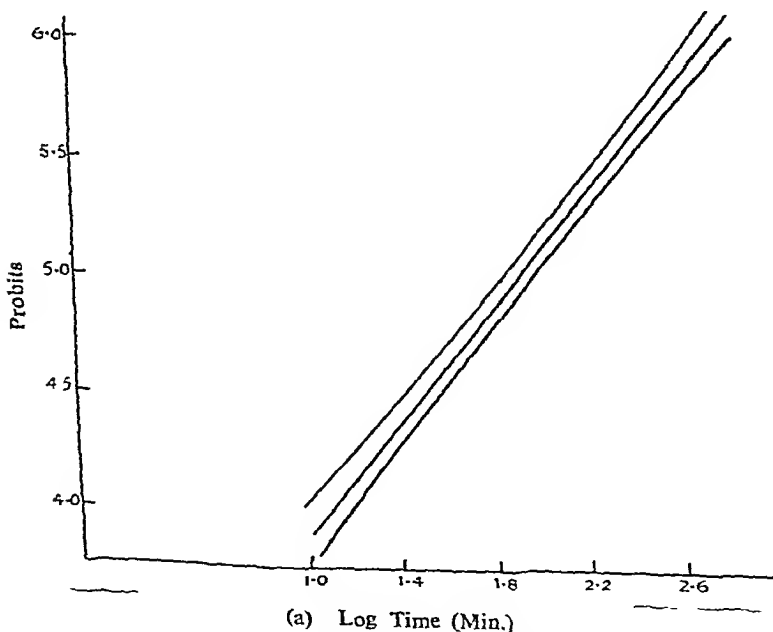


FIG. 1 (a).—Confidence limits (at $P = 0.05$) of probit-log time regression calculated from 32 expts. of 75 per cent. ethylene glycol at 20°C.

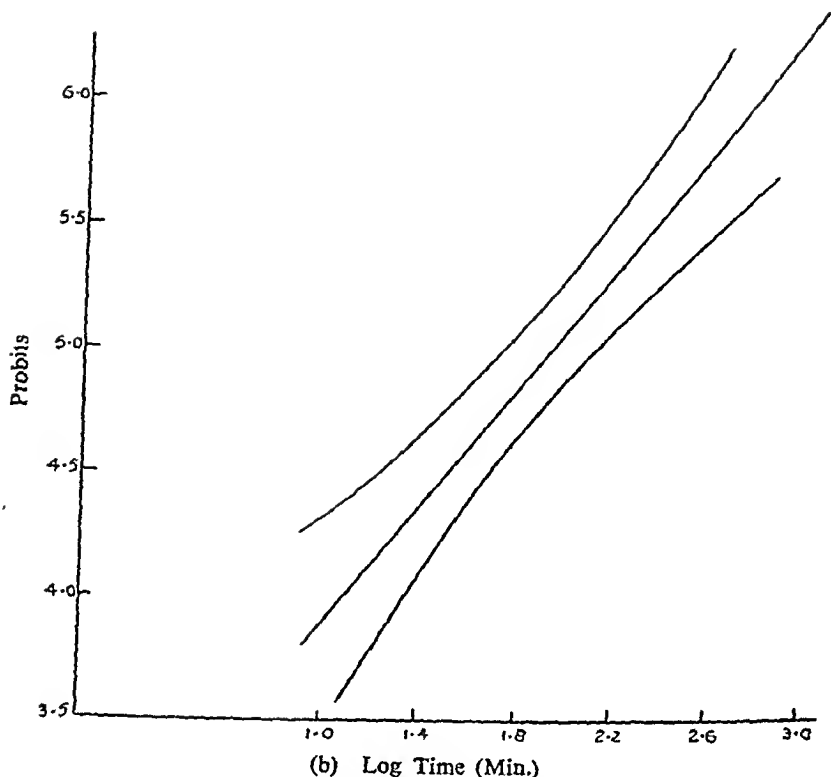


FIG. 1 (b).—Confidence limits (at $P = 0.05$) of probit-log time regressions calculated from 4 expts. of 0.425 per cent. ethylene glycol monohexyl ether at 20°C.

SUMMARY

1. The course of the disinfection (at 20°C) between *Bact. coli* and several concentrations of the following ethers of ethylene glycol has been investigated: monomethyl, monoethyl, monopropyl, monobutyl and monohexyl. Several experiments were conducted at every concentration and probit-log. time regressions calculated for all experiments.

2. For every concentration of a substance the sum of squares for the common regression and for the variation in regression was calculated; the error mean square of the regression was also computed.

3. The data for every concentration of each compound have been pooled, and a mean regression has been calculated for each compound.

4. The statistical data from all the calculations for the terms of the regressions for every concentration of the compounds (at 20°C) have been massed and an analysis of variance carried out.

5. The probabilities for the differences between the mean squares of the items in the analysis of variance have been deduced by means of the z distribution.

6. No significant difference could be shown between the variation in regression between concentrations and between tests; these two errors

have been pooled in order to establish the error mean square for all the estimations performed.

7. The probit-log. time regression coefficient for every compound has been compared with its standard error; in all cases the ratio was large thereby indicating that b had been estimated satisfactorily.

8. Confidence limits (at $P=0.05$) of the probit-log. time regressions have been calculated and a diagram constructed to show the increased precision obtained when many tests are performed at the same concentration.

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as indicator, the same final titration value was obtained whether the titration was completed with standard hydrochloric acid or standard ammonium thiocyanate solutions. The results of a number of titrations are recorded, using 3 drops of a 0.5 per cent. tartrazine solution, and taking the end-point at the stage when the dye was removed from the precipitate and the supernatant liquid assumed a rich lemon colour: agreement with the theoretical value was of the order of 1 in 3,000.

R. E. S.

PLANT ANALYSIS

***Courbonia Virgata*, Chemical Composition and Basic Constituents of.** A. J. Henry and D. N. Grindley. (*J. Soc. chem. Ind., Lond.*, 1949, 68, 9.) The chemical composition of the air-dry root of *Courbonia virgata* A. Brongn. (Capparidaceae) is:—moisture, 5.90 per cent., ash 7.47 per cent., protein 17.41 per cent., crude fibre 5.78 per cent., sucrose 33.53 per cent., pentosan (araban) 11.17 per cent., fat (soluble in light petroleum) 3.37 per cent., fats (soluble in chloroform but insoluble in light petroleum) 0.81 per cent., tetramethylammonium hydroxide 0.93 per cent., other non-volatile bases, about 0.03 per cent., and some dimethylamine and trimethylamine. The bases probably occur as chlorides. The composition of the ash, and the characteristics of the root fat have been determined. The seeds contain smaller quantities of fat, ash, protein and crude fibre, and the main carbohydrate is starch (64.86 per cent.) The principal basic constituent (tetramethylammonium hydroxide) is present in the proportion of 0.93 per cent. in roots, 0.78 per cent. in aerial stems, 0.70 per cent. in leaves, 0.65 per cent. in thick scaly shoots and 0.15 per cent. in seeds. The method for the isolation of tetramethylammonium hydroxide from the air-dry material is as follows. Percolate with alcohol, evaporate, extract with water, and filter to remove oily matter. Treat the filtrate with basic lead acetate, remove the excess with hydrogen sulphide, evaporate to small bulk, warm with water to 55° to 60°C., add iodine-potassium iodide solution, cool overnight and collect the precipitate on a sintered glass filter. Expel the free iodine by prolonged treatment with hot water, evaporate the solution, dry at 110°C, extract with cold water and filter. Evaporate the filtrate to dryness and wash with dehydrated alcohol until no more colour is extracted. Recrystallise from hot water and wash with dehydrated alcohol. Dimethylamine, trimethylamine and non-volatile bases can be extracted from the alcoholic washings.

G. B.

***Dichroa febrifuga* Lour, Antimalarial Constituents of Chinese Drug Ch'ang Shan.** T. Q. Chou, F. Y. Fu and Y. S. Kao. (*J. Amer. chem. Soc.*, 1948, 70, 1765.) Extraction of the powdered root yielded extracts which were found to contain umbelliferone, 4-quinazolone, a base with the composition $C_{18}H_{23}O_3N_3$ and a water-soluble alkaloid named dichroine. This had the composition $C_{16}H_{21}O_3N_3$ and isomerised readily under suitable conditions to three isomerides α -, β - and γ -dichroines. When oxidised with potassium permanganate, dichroine yielded 4-quinazolone among other products; hydrolysis with sodium hydroxide gave the decomposition products anthranilic acid, formic acid, and ammonia, together with a compound which behaved like a pyrrole derivative. Benzoylation gave a tribenzoyl derivative. Neither carboxyl-, methyl-, nor methylenedioxy-groups were detected. Dichroine formed both normal and acid salts and a nitroso compound. Regarding the antimalarial activity of dichroines, the

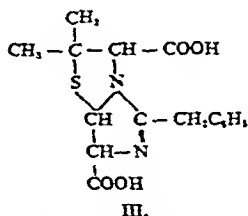
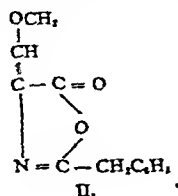
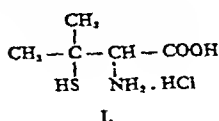
α -isomeride showed the greatest, and the γ -isomeride the least, the curative dose for chicken malaria being found to be 4 mg. of γ -isomer per kg.

R. E. S

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

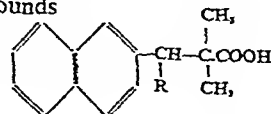
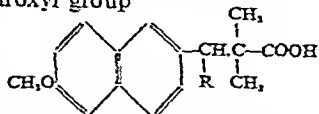
Benzylpenillic Acid, Synthesis of. R. W. Holley, F. H. Carpenter, A. H. Livermore, and V. du Vigneaud. (*Science*, 1948, 108, 136.) The condensation of D-penicillamine hydrochloride (I) with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (II) in pyridine containing triethylamine and subsequent treatment of the crude condensation product, gave needles of D-benzylpenillic acid (III) (micro m.pt., 180° to 182°C. (in 19 per cent. yield), optical rotation $[\alpha]_D^{20} + 471^\circ$ (0.1 per cent. in methyl alcohol); the benzylpenillic acid isolated was the same isomer as that obtained from



natural benzylpenicillin. When L-penicillamine hydrochloride was substituted for D-penicillamine hydrochloride in the procedure, L-benzylpenillic acid was obtained, identical with D-benzylpenillic acid in all respects, except for its opposite optical rotation, $[\alpha]_D^{21} - 476^\circ$ (0.09 per cent in methyl alcohol). When DL-penicillamine hydrochloride was used, optically inactive benzylpenillic acid was obtained (micro m.pt., 177°C. to 179°C). Reference is made to a small antibiotic activity found after the condensation of I and II in pyridine (containing no triethylamine) and to other data published in *The Chemistry of Penicillin*, Princeton Univ. Press, 1948.

R. E. S.

β -Naphthylpropionic Acid, Substituted, Oestrogenic activity of. A. Horeau, J. Jacques and S. Julia. (*C. R. Acad. Sci., Paris*, 1948, 227, 1278.) The suppression of a phenolic hydroxyl group generally results in a considerable diminution of the physiological activity of oestrogens. Substituted (methyl or ethyl)-naphthylpropionic acids (allenolic acids) of the type are known to be powerfully oestrogenic. The present paper describes the preparation of the corresponding non-phenolic compounds. These acids were found to be about 10 times less active than the methoxy compounds. Detailed biological results are to be published later.



G. M.

Suramin, Studies on. H. M. Dewey and A. Wormald. (*Biochem. J.*, 1948, 43, 24.) Experiments were carried out to determine whether any simple antiseptic could be added to blood serum or plasma to preserve it for subsequent suramin determinations without interfering with the colour reaction; at the same time an investigation into the reliability of suramin determinations on whole blood samples was made. Throughout the work the suramin was determined by hydrolysis, diazotisation and coupling with methyl- α -naphthylamine, the pink colour produced being matched against a standard Tintometer disc. In a number of determinations in which suramin was added to whole blood (ox, rabbit, and human) the recovery ranged from 60 to 97 per cent.: it was considered that this was due to constituents of the blood cells which inhibited diazotisation or coupling. Quantitative recoveries were obtained when differing amounts of suramin were added to hydrochloric acid-hydrolysed blood. When suramin was added to a sample of whole blood it gave 64 to 69 per cent. recovery in straight determinations but recovery values of 92 and 97 per cent. were obtained from the separated plasma and the red cells. The suramin was almost completely confined to the plasma. The addition of ether, chloroform, toluene (0.3, 0.2 and 0.2 ml. respectively per 2 ml. of serum); phenol and merthiolate (to give concentrations of 0.2 to 0.4 and 0.018 per cent. respectively) and 10.3N HCl (3 ml./2 ml. serum) to serum from rabbits injected with suramin, showed that ether, chloroform, toluene, and hydrochloric acid were not completely satisfactory as preservatives. Serum preserved with phenol or merthiolate gave satisfactory results over periods of 1 to 5 weeks.

R. E. S.

BIOCHEMICAL ANALYSIS

Bismuth in Biological Material, Determination of. E. P. Laug. (*Anal. Chem.*, 1949, 21, 188.) The direct extraction of bismuth with dithizone from aqueous solutions at pH 2 cannot be applied satisfactorily to acidified digests of biological material owing to the serious interference of halides and phosphates. It was found that in the presence of 20 per cent. acetic acid with carbon tetrachloride substituted for chloroform as the solvent for dithizone, quantitative extraction of bismuth could be made in the presence of phosphates and halides at pH 2.5; simultaneously a separation from lead was effected. Details of the method given involve dry-ashing of the sample at 500°C. followed by solution in concentrated nitric acid. To the diluted solution glacial acetic acid is added to pH 2.5 and bismuth is extracted with successive portions of dithizone in carbon tetrachloride; under these conditions some copper and zinc but no lead are extracted. The metal dithizonates are washed with dilute nitric acid and then with dilute nitric acid containing potassium bromide. By this means, bismuth dithizonate is decomposed and the bismuth reverts to the aqueous phase as a complex bromide salt. When the aqueous phase is adjusted to pH 9.5, this complex is again decomposed. Bismuth is then extracted with dithizone in chloroform and the density of the coloured solution is determined in a spectrophotometer at 490 μ . Results are given for the recovery of bismuth added to rat tissue

R. E. S.

Cadmium in Biological Materials and Foods, Estimation of. R. L. Shirley, E. J. Benne and E. J. Miller. (*Anal. Chem.*, 1949, 21, 300.) A procedure for estimating small amounts of cadmium is given. The sample is first evaporated to dryness with 2N sulphuric acid and ashed at

550°C.; the residue is then extracted with N/1 hydrochloric acid, the resulting extract being diluted, neutralised, and adjusted to pH 2.0 to 2.3. The adjusted acid extract is then treated as follows:—(1) an aliquot portion is extracted with a carbon tetrachloride solution of dithizone to remove the interfering ions of copper and mercury and most of the cobalt and nickel, (2) the aqueous phase is adjusted to pH 8.5 to 9.0 with an ammonium hydroxide-ammonium citrate buffer solution containing Rochelle salt, dimethylglyoxime is added, and the solution is extracted with chloroform to remove cobalt and nickel not extracted previously, (3) sodium hydroxide is added to make an approximately 5 per cent. solution followed by extraction with a carbon tetrachloride solution of dithizone to remove the cadmium. The extracted cadmium dithizonate was determined photometrically, the maximum absorption occurring at 515 m μ . The use of amber glassware was found to give protection from light, particularly in the case of the blank solution. Results of the sensitivity of the procedure compared favourably with those published previously. Amounts of magnesium or calcium phosphate greater than 100 mg. may interfere.

R. E. S.

Pregnandiol, New Colorimetric Determination of. J. W. Goldzieher. (*J. Lab. clin. Med.*, 1948, 33, 251.) An aliquot portion, containing about 0.1 to 1 mg. of pregnandiol, of a solution of the sample in a suitable solvent such as ether-alcohol is evaporated to dryness in a 10-ml. graduated flask, and about 6 ml. of zinc chloride reagent (38 per cent. in glacial acetic acid) and exactly 2.5 ml. of acetyl chloride added. The flask is warmed in a water-bath at 50°C. for 30 minutes, cooled in ice to room temperature, allowed to stand for 20 minutes, and the volume adjusted to 10 ml. with zinc chloride reagent. The intensity of the colour is compared with that of a standard prepared in the same manner using 0.5 mg. of pregnandiol. The method was found to be accurate to within 4 per cent.

G. R. K.

PHARMACY

DISPENSING

Pectin of *Opuntia vulgaris*, Penicillin delay action of. H. Diacono and V. Massa. (*Ann. pharm. Franc.*, 1949, 6, 461.) The addition of the pectin of *Opuntia vulgaris* to penicillin solution gives a delaying effect comparable to that of Subtosan (which contains polyvinylpyrrolidone). The formula used was as follows. Calcium magnesium pectate from *Opuntia vulgaris*, 10 g.; sodium chloride, 8.5 g.; potassium chloride, 0.5 g.; calcium chloride cryst., 0.5 g.; magnesium chloride cryst., 0.005 g.; N/1 hydrochloric acid, 17.1 ml.; sodium bicarbonate, 1.68 g.; water, to 1 l. Repeated intramuscular administration of 0.1 g. of this pectin to guinea-pigs did not produce any harmful effects.

G. M.

Procaine and Procaine-Adrenaline, Stability of Solutions of. F. Gélébart. (*Ann. pharm. Franc.*, 1949, 6, 439.) Solutions of procaine may become discoloured on storage, owing to oxidation. This may be prevented by the addition of sodium bisulphite. When adrenaline is also present, the procaine appears to catalyse the oxidation of the adrenaline, and discoloration is much more rapid. In such solutions the physiological action of the adrenaline is reversed, the action being hypotensive, while the procaine, in addition to losing its anæsthetic action, acquires an increased toxicity while

sensitising the patient to the toxic action of the changed or unchanged adrenaline. Impurities in the procaine are an important factor in producing this decomposition, and ampoules prepared with pure procaine and distilled water free from traces of metals may be kept for several months, even without the addition of sulphite.

G. M.

PHARMACOGNOSY

Colchicum Seeds, Colchicine Content of. F. Santavý and J. Buchniček. (*Pharm. Acta Helvet.*, 1949, 24, 20.) The colchicine content was determined in 111 samples of the seeds of *Colchicum autumnale* Linn. from Moravia and Silesia. It was found to vary from 0.60 to 1.23 per cent.; the mean value being 0.81 per cent. The weight of the seeds varied from 183 to 406/g., the mean value being 262. The colchicine content reached a maximum (1.2 per cent) with seeds corresponding to 350/g.: with larger or smaller seeds the percentage was less.

G. M.

Datura, Effect of Cultural Conditions on Alkaloidal Content of. R. Hegnauer and H. Flück. (*Pharm. Acta Helvet.*, 1949, 24, 1.) Two kinds of *Datura* grow well in Switzerland, and these have been regarded as separate species, the white flowered form being *D. stramonium* Linn. and the violet flowered one as *D. tatula* Linn. A detailed description is given of the morphology and biology of the former species. There is a considerable difference in the alkaloidal content of individual plants, even though all are seedlings of a single mother plant. The alkaloidal content also varies with the variety, e.g., *D. stramonium* "Zurich" contained, in the leaf, from 0.128 to 0.235 per cent. of alkaloid; while *D. stramonium inermis* "Dordrecht" had from 0.335 to 0.578 per cent. The mean alkaloidal content of seedlings varied in the same way as that of the mother plants, indicating that yields can be improved by selection. Removal of the capsules or, even more, of the flowers, leads to a greater vegetative development with, at the same time, an increase in the percentage of alkaloids. The attempt to produce the same effect by spraying the plants with growth hormones resulted in a considerable disturbance in the growth of the young flowers and leaves. The vegetative period of the treated plants was considerably lengthened, and the autumnal drop in alkaloidal content of the leaves was retarded.

G. M.

Psyllium Seeds and Their Mucilages, A Quantitative and Qualitative Evaluation of Official and Unofficial Species. D. Greenberg. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 139.) A convenient means for the quantitative determination of the mucilage separated from psyllium seeds has been described. This involves soaking the seeds for 20 hours, at first with vigorous agitation, in 20 ml. of water per g. of seeds and subsequent expression of the mucilage by hand press; the mucilage is dried at 95° to 100° C., and weighed. The swelling factor of psyllium seeds is not indicative of their mucilage content, but depends on the viscosity of the mucilage formed, and other related factors. The viscosity, the swelling factor, and the proportion of mucilage obtained show no direct relationship between the many possible swelling factors obtainable for any given lot of seeds and the proportion of mucilage yielded by the seeds, nor is the proportion of mucilage obtainable from the seeds an indication of the viscosity of that mucilage; it is therefore suggested that more exacting standards than now required by the N.F. VIII

for the evaluation of psyllium seeds be considered. Two unofficial seeds have been described and assayed. *Plantago rhodosperma* grows on sandy soil from Missouri and Oklahoma to Louisiana, Texas and Arizona; the seeds yield 16 to 20 per cent. of a very thick and viscous mucilage, 150 mg. of which diluted to 20 ml. with water yields a semi-solid mass, resembling closely the consistency of "set" gelatin. *P. wrightiana* grows in dry sandy soil and is a native of Texas and Arizona; the seeds compare favourably in all respects with the official seeds, and yield about 20 per cent. of a viscous mucilage. These seeds of *P. wrightiana* can resist fermentation for several weeks, before yeast colonies begin to develop; they even resist transplants of mycelial mats of *Aspergillus niger*. This seems to indicate that there is present in *P. wrightiana* a substance which prevents or retards the growth of bacteria and fungi. The seeds of other species ferment within 24 to 48 hours after adding water, and become covered with moulds. L. H. P.

PHARMACOLOGY AND THERAPEUTICS

Antacid Buffers, A Study of, II. Prolonged Neutralisation. J. M. Holbert, N. Noble, and I. W. Grote. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 292.) An efficient antacid should neutralise the gastric acidity rapidly to pH 3.5 to 4, thus performing a double role in the prompt relief of pain and in the inactivation of pepsin, and should not cause reflex secretion of further hydrochloric acid by the gastric mucosa. In the evaluation of antacids the length of time required for neutralisation, the change in pH, and the length of time a given quantity of antacid is effective in neutralising acidity, must be considered. This length of time during which various antacids were effective in neutralising artificial gastric juice was studied *in vitro* under conditions resembling those in the stomach, the acid reaction mixture was removed from time to time, and an equivalent volume of fresh and artificial gastric juice was added. The efficiency of the antacid was followed by pH determinations. This method is a modification of the procedure due to Johnson and Duncan (*Quart. J. Pharm. Pharmacol.*, 1945, 18, 251.); 10 commonly used antacid powders were compared, they included 2 preparations of aluminium hydroxide, aluminium dihydroxyaminoacetate, magnesium trisilicate, magnesium hydroxide, sodium bicarbonate, and several compound powders; the aluminium preparations appeared to be the antacids of choice, according to the above criteria. L. H. P.

Caronamide, Absorption and Elimination of. K. H. Beyer, E. K. Tillson, H. F. Russo, G. S. Schuchardt and S. R. Gass. (*J. Pharmacol.*, 1948, 94, 167.) Caronamide is rapidly and completely absorbed when administered orally to dogs, as judged by the plasma levels and the overall urinary recovery of the drug and its metabolite(s). Some 43 per cent. of a given dose of caronamide is excreted within 4 hours after its administration, and urinary recovery is essentially complete within 24 hours. After oral or intravenous administration of caronamide to normal dogs, or intravenous administration to bilaterally nephrectomised dogs, a portion of the drug is metabolised. About 60 per cent. of a dose of the drug administered to normal dogs is excreted as such, the remaining 40 per cent. being excreted in the form of an unknown metabolite or metabolites more soluble than the parent compound. The distribution of caronamide in the body is of the same order as, but greater than, that for mannitol, the volume distribution approximating extracellular water. S. L. W.

PHARMACOPŒIAS AND FORMULARIES

DANISH PHARMACOPŒIA, 9TH EDITION, 1948

THE new edition of the Danish Pharmacopœia consists of more than 1600 pages and is divided into three volumes. The first deals with general determinations, methods of investigation, reagents, etc., the second contains monographs on individual substances, and the third contains formulæ and general monographs for forms of medicaments (extracts, tablets, etc.) formerly included in the *Dispensatorium Danicum* and the *Pharmacopœia*. The third part is necessarily large because of the wide scope of the dispensing carried out in Danish pharmacies.

The distribution of the remainder of the contents of the pharmacopœia between volumes I and II has been necessitated by the increased knowledge of the drugs described and by the improvements in analytical control. Limit tests, determination of physical constants, quantitative methods, bacteriological tests and semi-micro tests are set out in a special section in volume I together with other important directions. Melting-point determinations of derivatives are often used for the identification of organic compounds. For inorganic compounds, distinguishing tests are often prescribed which exclude the presence of substances which give the same reaction as the substance which is to be identified. The number of limit tests carried out by comparison with a standard solution has been much extended. Most of the tests have been rewritten on the basis of investigations carried out in the laboratory of the Danish Pharmacopœia Commission to determine the effect of conditions such as method of mixing, acidity and temperature on the results. Assays are prescribed for nearly all compounds, also for a large proportion of the galenical preparations and for many vegetable drugs.

Although some vegetable drugs have been omitted from the new pharmacopœia, the number is still large and includes some less familiar products such as sweet almond seed, althæa, fig, gall and manna. The macroscopic and microscopic descriptions are fully detailed and include many new observations.

In Denmark specialities are controlled by the State Laboratory, and vaccines and sera, etc., are prepared by the State Serum Institute. The Danish Pharmacopœia, therefore, does not contain specifications for drugs which are only dispensed in the manufactured form.

Only three monographs for hormones are included and there are none on biological drugs. The hormones are adrenaline, stilbæstrol and stilbæstrol dipropionate. The vitamins cover the same range as in the British Pharmacopœia, 1948. Methods of biological investigation, which are continually developing, are not described but are left as the responsibility of the Danish Ministry of Health so that they may easily be changed when necessary.

Veterinary drugs are not described in this Pharmacopœia, but it has now been decided that the compounds and drugs which are used for the preparation of veterinary medicines shall, in future, be included, and a veterinary supplement is in the press.

The Latin names of certain drugs differ from those in use in England. The more important differences include: *ætheroleum* for essential oils, *acidum amygdalicum* for mandelic acid, *bolus alba* for kaolin, and *diæmalum*, *enhexymalum*, *hexemalum* and *phenemalum* for barbitone, hexo-

barbitone, cyclobarbitone and phenobarbitone respectively. Dextrosum hydrotum is called glycosum and phenytoin, phenantoinum. Four sulphonamides are included:—sulphadiazine, sulphamerazine, sulphanilamide and sulphathiazole. In addition to the barbiturates mentioned above there are monographs for diallylbarbituric acid (diallylmalum, allobarbitone) and allylpropylbarbituric acid (allypropymalum). The local anæsthetics are tetracaine (amethocaine), benzocaine, cinchaine hydrochloride (cinchocaine) and procaine.

Galenical preparations are treated very fully and there are general monographs for forms of medicaments which are less common in England, such as concentrata vegetabilium, dosipulveres, granulata and succi. Distinctions are made between suppositoria and vagitoria, between tablette (for internal use), injectablette and solublette (for solutions other than injections), tablette orales (for sucking, with local action in the mouth and throat) and trochisci (for chewing). For liquid preparations such names as injectabilia, oculoguttæ, mixturæ (used by dessert- or table-spoonfuls), liquors (used by tea-spoonfuls) and guttæ are used; the three last-mentioned names may be used only for preparations for internal use.

Eighty monographs for tablette are given but, in general, it is not required that technical details shall be followed exactly, merely that the content of drug and the form and size of tablet shall be as prescribed. A disintegration test is directed to be made on three tablets which should appreciably disintegrate after ten minutes shaking in water at 38° to 40°C. in a flask. In the general monograph on pills, directions are given for obtaining uniformity of appearance whether they are prepared by rolling out, extrusion with subsequent cutting off and rounding, or by compressing in a tablet machine followed by coating. A new system is used for the requirements for accuracy of dosage in tablets. The changes of requirements by steps found in, among others, the British Pharmacopœia and Danish Pharmacopœia 1933, are now avoided. For example, for tablets which weigh over 0.08 g. at least 30 are weighed and of these 90 per cent. must not deviate from the mean weight by more than 0.004 g. + 5 per cent. of the mean weight.

The twelve official eye-drops are prepared aseptically and the injections (67) are sterilised. Both forms of medicament must be isotonic with the liquids of the tissues. In volume I there are isotonic curves for a large number of compounds, compiled by Pedersen-Bjergaard and co-workers. Six methods of sterilising injections are given:—filtration, heating in steam or boiling water at 100°C. for 15 minutes or 1 hour, autoclaving at 120°C. for 20 minutes and a dry heat at 140°C. for 3 hours or at 160°C. for 2 hours. Injections not official in the British Pharmacopœia are:—tetrapon (opium alkaloids), sodium thiosulphate, sulphur, sodium iodide and sodium nitrite. Injection of aneurine hydrochloride is given in two strengths—1 per cent. and 2.5 per cent. Injection of glucose is prescribed as 10 per cent. and 50 per cent.

The stability of drugs has been carefully considered and the rules for heat treatment and time of storage, which are largely based on Danish investigations, are summarised in a Table in Volume I.

While the earlier editions of the Dispensatorium Danicum (1934 and 1938) had the form of a pocketbook and were intended for the use of both doctor and pharmacist, Volume III of the Pharmacopœia is mainly intended for pharmacists. The volume has the same format as the other volumes of the

(Continued on page 500)

LETTERS TO THE EDITOR

Vitamin B₁₂

SIR,—Our independent isolation of crystalline anti-pernicious anæmia factor from liver was announced^{1,2} within a few weeks of the initial American publication³ on Vitamin B₁₂. The identity of these two red crystalline preparations was presumed at the time, and has since been amply confirmed. We first argued, very tentatively, from determinations made on an ultra-micro scale, that the molecule contained three atoms of phosphorus. We have now known for some time that this was not so; the true value of 2.1 per cent. of phosphorus corresponds with one atom per molecule. We have corrected our original statement in a paper at present awaiting publication: although we did not think the matter worthy of separate record in the present conditions of scientific publications, we mentioned it widely to interested parties. Nevertheless unnecessary confusion may be created by a recent paper⁴ in your journal; its authors have seized upon this one apparent discrepancy to suggest that our material differs from their preparation of vitamin B₁₂.

We have been able to exchange samples with Messrs. Merck and Co. Inc., of Rahway, New Jersey, and we have made a detailed comparison of the materials. Our crystalline anti-pernicious anæmia factor is identical with their vitamin B₁₂ in the following respects:—cobalt and phosphorus* content; microbiological activity; ultraviolet and visible absorption spectra; single crystal X-ray diagrams†; refractive indices‡; polarographic step; behaviour on paper chromatograms; clinical potency.

The B.D.H. workers have thus confirmed our own finding, and that of the Merck workers, that the molecule of B₁₂ contains one atom each of cobalt and phosphorus.

E. LESTER SMITH.

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Glaxo Laboratories Ltd.
Greenford, Middlesex.

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1. Lester Smith and Parker, *Biochem. J.*, 1948, 43, Proc. viii.
2. Lester Smith, *Nature*, 1948, 162, 144.
3. Rickes, Brink, Koniuszy, Wood and Folkers, *Science*, 1948, 107, 396.
4. Ellis, Petrow and Snook, *J. Pharm. Pharmacol.*, 1949, 1, 287.

* Determined by Mr. S. Trippett and Dr. S. F. Macdonald at the University of Cambridge.

† Determined by Dr. D. Hodgkin at the University of Oxford.

‡ Determined by Mr. R. C. Spiller at the University of Oxford.

PHARMACOPŒIAS AND FORMULARIES (Continued from page 499)

pharmacopœia, but since the pharmaceutical directions are made fuller than before and there are also included analytical requirements for a large number of preparations, the book is not particularly suitable for the use of medical men. The Pharmacopœia Commission has therefore produced a book specially for doctors. In this book, *Pharmaconomia Danica*, which will be published shortly, the compositions of these preparations of the pharmacopœia which are of use to the doctor are explained, together with the actions, uses and doses.

BOOK REVIEWS

THE PRINCIPLES OF BIOLOGICAL ASSAY, by C. W. Emmens. Pp. 204 and Index. Chapman and Hall, Ltd., London, 1948. 21s. 0d.

As Sir Percival Hartley says in the opening words of his introduction to this work "biological assay is now a recognised tool for the study of certain properties of living matter." The use of techniques of biological assay has increased from that of standardisation of drugs, important and expanding in scope as that work may be, to include methods of biological assay as a tool of research. The worker in this field must ever hold clearly before his mind's eye the facts that biological assays are of necessity conducted by comparing the unknown with a standard preparation of the same substance, and that the methods of comparison are such that the dominant factor of biological variation may not be excluded. This volume has been written with these precepts in mind. The author emphasises the duty of the experimenter not merely to analyse his data by known statistical methods but to plan his experiment beforehand so that the results are readily capable of analyses by recognised methods. It is fitting that such a clear, concise and stimulating volume should appear from the National Institute for Medical Research, Hampstead, which has been the site of so much endeavour in this field. The reader will not find in the contents list or the index of this book any mention of a recognised method of assay, or of any substance which is normally subjected to assay—such topics are adequately treated elsewhere—but he or she will find in these pages a readable, understandable and thought-provoking account of the principles underlying the choice and arrangement of experimental designs which may be expected to yield the optimum quantity of useful information, and of the statistical methods of analysing that information so as to extract the demonstrable truth from it. This is a book for the advanced student of biological assay methods, for the teacher, research worker, and planner of laboratory procedures. If one may plagiarise Lord Kelvin's dictum that "mathematics is like a mill, one cannot take out more than one has put in" this book should stimulate workers so to design their investigations that more is "put in" and therefore one may reasonably hope that more may be "taken out." Common fallacies in procedure are emphasised and undoubtedly any worker who reads and digests this volume will benefit greatly. This may be termed a book of statistics "with a difference."

J. D. P. GRAHAM.

LA CHIMIE DES VITAMINES ET DES HORMONES, by Joseph Sivadjan. Vol. 1. Pp. 479 and Index. Gauthier-Villars, Paris (6^e), 1949.

The third edition of this book is published in two parts, the first being devoted to Vitamins and the second to Hormones. The writing of Part I was completed in June, 1946, but owing to difficulties in publication (at least as great in France as elsewhere), it has only just appeared (1949). It cannot therefore contain accounts of work published since 1946; e.g., the synthesis of vitamin A and the latest work on folic acid, biotin and pantothenic acid. On the other hand, the chemistry of vitamins E, K, C., riboflavine, aneurine, pyridoxine and nicotine acid is established. New chapters have been added in this edition on the antihæmorrhagic factors, the acrodynia factor and the antipellagrous factor, biotin, pantothenic acid, essential fatty acids and folic acid. Well-known substances such as choline, *p*-aminobenzoic acid and

BOOK REVIEWS

inositol whose vitamin-like functions have been recognised since their chemical nature became well known, are not discussed. Each chapter is a compilation of published information on the vitamin concerned from the time its existence was first recognised up to the time of writing. Much of this is interesting from an historical point of view; e.g., Rosenheim's first use of arsenic trichloride as a colour test for vitamin A, but there is a complete lack of discrimination between good and bad methods. All the "Units" of vitamin A which have ever been used are given and their relationships one with another as claimed by various workers, but there is no indication of the fact that most of these relationships do not (in fact cannot) hold, and a good opportunity of explaining why this is so has been missed. For the discriminating reader this should be a valuable book of reference.

K. H. COWARD.

BOOKS RECEIVED

MINOR SURGERY edited by Sir Heneage Ogilvie and W. A. R. Thompson. Pp. 176 and Index, Eyre and Spottiswoode Ltd., London, 1949, 2nd edition, 14s.

THE CHEMISTRY OF PENICILLIN edited by H. T. Clarke, J. R. Johnson and Sir Robert Robinson. Pp. 1042 plus Index and Appendix, Princeton University Press (London: Geoffrey Cumberlege), 1949, £9 9s.

AIDS TO FORENSIC PHARMACY by M. S. Bolton. Pp. 226 and Index, Bailliere, Tindall and Cox, London, 1949, 4th edition, 6s.

UNIVERSITY COLLEGE HOSPITAL PHARMACOPŒIA, 1949, edited by T. D. Whittet.

PHARMAKOLOGIE DES DEUTSCHEN ARZNEIBUCHS 6. UND DES ERGANZUNGSBUCHES 6 by H. Braun. Pp. 344 and Index, Wissenschaftliche Verlagsgesellschaft mbH., Stuttgart, 1949, 3rd edition.

'ANALAR' *STANDARDS FOR LABORATORY CHEMICALS* formulated and issued jointly by The British Drug Houses Ltd. and Hopkins and Williams Ltd., London, 1949, 4th edition, 10s. 6d.

NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Asma-Vydrin* is a spray solution for use by oral or nasal inhalation. It contains atropine methylnitrate 0.14 per cent., papaverine hydrochloride 0.88 per cent., pituitary extract 0.75 per cent., chlorbutol 0.50 per cent., and adrenaline 0.55 per cent. It is indicated in the symptomatic treatment of asthma, emphysema and chronic bronchitis associated with bronchial spasm. It is administered as an aerosol by means of an oro-nasal inhaler, the spray being inhaled for 1 or 2 minutes whenever an attack threatens or at the onset of an attack. It may be used every 2 or 3 hours, if necessary, without producing toxic effects or habituation. The pH of the solvent is adjusted to avoid the production of irritation after prolonged use. The inhalation liquid is issued in bottles containing $\frac{1}{2}$, 1 and 4 fl. oz. S. L. W.

Cetyl Pyridinium Chloride. (*New and Non-official Remedies, J. Amer. med. Ass.*, 1948, 137, 701.) Cetyl pyridinium chloride is the monohydrate of the quaternary salt of pyridine and cetyl chloride, and contains not less than 97 per cent., and not more than 103 per cent., of $C_{21}H_{35}NCl \cdot H_2O$; mol.wt. 357.99. It occurs as a white powder; odour, slight; melting-range, 77°C. to 83°C. Very soluble in water, alcohol and chloroform; only slightly soluble in ether and benzene. A 1 per cent. aqueous solution has pH 6 to 7 when determined by indicators, but glass-electrodes give variable results. Surface tension of a 1 per cent. aqueous solution at 25°C., about 10.40, and of a 10 per cent. solution 38.15. When dried to constant weight *in vacuo* over phosphorus pentoxide, loses not less than 4.5 per cent. and not more than 5.5 per cent. of its weight; ash, with reference to the sample dried to constant weight, not more than 0.2 per cent. It gives the reactions characteristic of chlorides. On melting, it becomes brown and evolves the odour of pyridine. On adding 5 ml. of 0.01M potassium ferricyanide to 5 ml., a yellow precipitate is produced. 1 ml. of a saturated solution of potassium thiocyanate produces with 1 ml. a white gelatinous precipitate, and 1 ml. of a saturated solution of picric acid produces with 1 ml. a yellow precipitate. For the assay, dissolve 0.25 g., accurately weighed, in 5 ml. of a buffer solution containing 26 per cent. of sodium acetate and 9 per cent. of acetic acid, add 50 ml. of 0.1M potassium ferricyanide and make the solution up to 100 ml. with water. Mix, allow to stand for 1 hour, and filter, rejecting the first 15 ml. of filtrate. To 50 ml. of the remaining filtrate, add 5 ml. of a 10 per cent. solution of potassium iodide in water, and 10 ml. of hydrochloric acid; allow to stand for 1 minute. Add 10 ml. of a 10 per cent. solution of zinc sulphate in water, and titrate with 0.01 N sodium thio-sulphate, using a starch test-solution near the end-point: each ml. of 0.01 N sodium thiosulphate is equivalent to 0.01074 g. of $C_{21}H_{35}NCl \cdot H_2O$. Cetyl pyridinium chloride is a cationic detergent, possessing antiseptic as well as useful surface-active properties. It is incompatible with anionic detergents, such as soaps, and may be reduced in the presence of serum and tissue fluids. It is not reliable against clostridial spores. L. H. P.

Dihydroxy Aluminium Aminoacetate. (*New and Non-official Remedies, J. Amer. med. Ass.*, 1948, 137, 1226). Dihydroxy aluminium aminoacetate is a basic aluminium salt of glycine containing small amounts of aluminium

NEW REMEDIES

hydroxide and glycine; $C_2H_6O_4NaI$, mol. wt. 135.05. It is a white powder, odourless, taste slightly sweet; insoluble in water and in organic solvents, soluble in dilute mineral acids and fixed alkalis forming a cloudy solution which clears on heating. Loses, when dried to constant weight at $130^{\circ}C$. for 2 or 3 days, not more than 14.5 per cent. of its weight. A 4 per cent. w/v suspension in distilled water has pH 6.5 to 7.5; a 0.8 per cent. w/v suspension in 0.1N hydrochloric acid has pH above 3.0. On adding excess of ammonia solution to an acidified 4 per cent. w/v suspension, a white flocculent precipitate, insoluble in excess of ammonia but soluble in sodium hydroxide solution, is produced. On adding one drop of liquefied phenol and 5 ml. of sodium hypochlorite solution to 10 ml. of a similar acidified suspension, a blue colour, characteristic of aminoacetic acid, is produced. The compound must be free from heavy metals and from mercury; when a 5 per cent. suspension in 0.1N potassium permanganate acidified with concentrated sulphuric acid is refluxed for 30 minutes, the distillate must be free from acetone. At room-temperature 1 g. neutralises in 10 minutes not less than 125 ml. and not more than 175 ml. of 0.1N hydrochloric acid, using bromophenol blue solution as indicator. Dihydroxy aluminum aminoacetate contains not less than 9.8 per cent. and not more than 10.8 per cent. of nitrogen, when determined by the semi-micro Kjeldahl method of the U.S.P., and not less than 34.9 per cent. and not more than 38.7 per cent. of Al_2O_3 , when determined by a method depending on ignition with acid, solution of the aluminium salts formed, precipitation of the hydroxide and ignition to form the oxide.

L. H. P.

Disprin* tablets contain 5 gr. of acetylsalicylic acid with the requisite calcium base to form, in water, 6 gr. of calcium aspirin. The tablets have the advantage over ordinary calcium aspirin tablets that the calcium aspirin is not formed until the tablets are dissolved in water, that is, until it is actually required for use. The tablets are therefore much more stable, less liable to contain free salicylic acid, and less liable to cause gastric irritation than calcium aspirin tablets. Disprin tablets are used for the same purposes as aspirin, the average adult dose being 2 tablets repeated four-hourly. S. L. W.

Heptalgin* is 4 : 4-diphenyl-6-morpholinoheptan-3-one hydrochloride, and is an analgesic with a potency about 6 times that of morphine, 20 times that of pethidine, and 3 times that of amidone, and having an acute toxicity in relation to analgesic potency much lower than any of these substances. Given orally it exerts its effect within 15 to 30 minutes, relief from pain lasting from 3 to 4 hours; with parenteral administration the effect commences within 2 or 3 minutes and lasts for 1 or 2 hours. It abolishes pain with little or no accompanying cortical depression. Apart from mild drowsiness following full dosage, it does not give rise to hypnotic effects, and it has only a slight depressant action on the respiratory centre. Early experience suggests that addiction is improbable, and there is no evidence of development of tolerance. In a small proportion of cases it may cause transient giddiness; other minor side-effects are rare. In clinical trials, it has given relief from pain in fibrositis, pleurisy, and coronary thrombosis; in the headache of subarachnoid hæmorrhage; in gall-bladder and ureteric colic; in inoperable malignant growth; in sinusitis, toothache, and gastric ulcer. Dosage varies from 10 to 30 mg. by mouth, the dose by subcutaneous or intramuscular injection being 10 mg. in 1 ml. Tablets containing 10 mg. are issued in bottles of 25 and 100, and ampoules containing 10 mg. in 1 ml. in boxes of 6.

REVIEW ARTICLE

SCLERENCHYMA IN THE DIAGNOSIS AND ANALYSIS OF VEGETABLE POWDERS

BY T. E. WALLIS.
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Curator of the Museum of The Pharmaceutical Society of Great Britain

INTRODUCTION.

SCLERENCHYMA is the name given to any hard vegetable tissue other than vascular tissue. There are two types of sclerenchyma, viz.:—(a) parenchymatous sclerenchyma, the cells of which are termed sclereids or stone-cells, and (b) prosenchymatous sclerenchyma, the cells of which are termed fibres. Both sclereids and fibres have heavily thickened walls which are usually lignified; in a few plants, however, cellulosic sclerenchyma occurs, as in the endosperm of the date, *Phœnix dactylifera* Linn., and in that of the corozo or vegetable ivory nut, *Phytelphas macrocarpa* Ruiz et Pav., which are composed of sclereids, and in the pericycle of flax and hemp and the phloem of mezereon and slippery elm barks, in which the fibres are unlignified. The striking appearance and, when lignified, the strong staining reactions of these cells render them easily identifiable and, since as long as a century ago, they have been regularly used as a means of identifying vegetable materials, such as tea (sclereids) and cinchona barks (fibres), (see Fig. 1 R and G).

The identifications were based at first upon the form, abundance and manner of distribution of the sclerenchyma. Measurements were not usually given for sclereids and only rarely for fibres, although they were used for starches and blood-corpuscles and for materials like lupulin and lycopodium which are composed of discrete particles. Towards the end of the nineteenth century measurements of the length and width of cells in sclerenchyma began to be made as a routine addition to the verbal descriptions. The dimensions given were used chiefly as a record of observed facts and also for the purpose of making drawings to scale. Greenish (1903) in his "Foods and Drugs" and Greenish and Collin (1901 to 1904) in their "Anatomical Atlas" advocate and use measurements for starches, but only rarely for crystals of calcium oxalate, e.g., in rhubarb, squill, and orris, and very occasionally for the dimensions of cells; otherwise only vague statements such as "very large cells", "short hairs", "small rosettes", etc., are made about dimensions. Tschirch and Oesterle (1890) introduced many measurements into their "Anatomischer Atlas" and, about the same date, measurements began to be used for the characterisation of certain drugs, such as the different cinchonas and for the exclusion of particular adulterants, such as cassia bark when substituted for cinnamon. Ludwig Koch in his atlas "Die mikroskopische Analyse der Drogenpulver" 1900-1908 made a still more systematic use of dimensions of cells and carefully recorded the linear measurements of all the structures present in the powders and drugs he

examined. These dimensions, however, considered as criteria of purity, are not very satisfactory, since they fail to exclude quite large percentages of adulterants and, moreover, may be indecisive when attempting to establish merely the identity of a powdered drug.

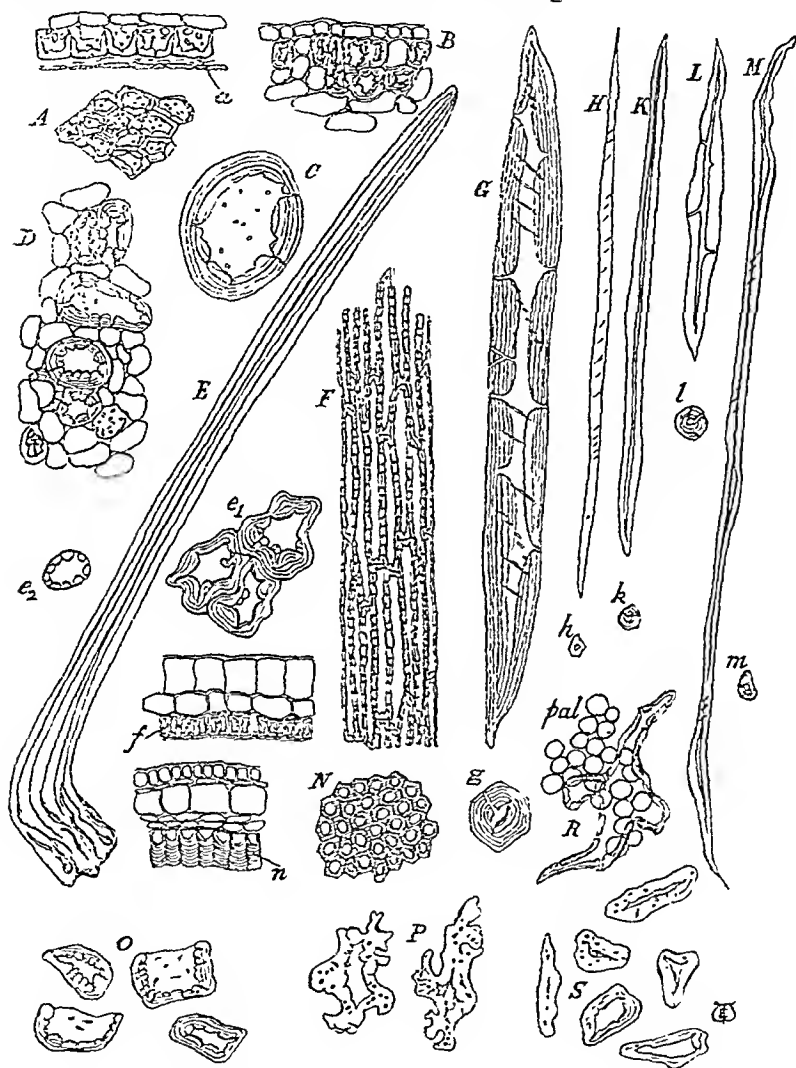


FIG. 1.—Typical Sclereids and Fibres. all $\times 150$. Sclereids of A. beaker-cell layer of *Piper nigrum* Linn., a, the same in section; B. hypodermal layer of *Piper nigrum* Linn., C. *Juniperus plantcea* Linn., D. *Eugenia caryophyllus* (Spreng.) Sprague; E. *Strychnos nux vomica* Linn., e₁, section of trichome bases; e₂, section of limb of trichome; F. *Litum usitatissimum* Linn., f, the same in section; N. *Eleteria cardanum* Maton var. *minuscula* Burkill, n, the same in section; O. *Cinnamomum zeylanicum* Nees; P. *Viburnum prunifolium* Linn.; R. *Camellia sinensis* (Linn.) O.Ktze. pal, palisade cells; S. *Rhamnus purshianus* D.C.; G. Fibres of *Cinchona succirubra* Pav.; H. *Rhamnus purshianus* D.C.; K. *Cinnamomum zeylanicum* Nees; L. *Sassafras variifolium* (Salisb.) O. Kuntze; M. *Quillaia saponaria* Molina; g, h, k, l, m transverse sections of the corresponding fibres.

SCLERENCHYMA IN DIAGNOSIS OF POWDERS

SCLEREIDS PER SQUARE MILLIMETRE.

When the cells to be measured form a continuous layer one cell in thickness, it is possible to make an improvement upon simple linear dimensions; this is effected by counting the number of cells per sq.mm. of the layer. The values so obtained provide an automatic averaging of the breadth and length of a very large number of cells, thus yielding data which can replace or supplement linear measurements, the ranges of which frequently overlap so much as to give inconclusive results, when such measurements are used to differentiate between similar tissues derived from closely related plants. The improvement effected has been demonstrated for the sclerenchymatous layer of the testa of various types

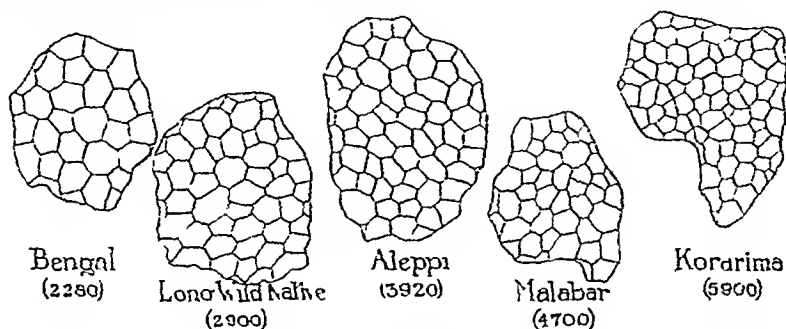


FIG 2—Outline of cells in typical fragments of sclerenchymatous layer of cardamom seeds ($\times 175$)

of cardamom seeds (Fairbairn¹). The drawings in Figure 2 show how difficult it is to make distinctions by the measurements of the diameters of individual cells, but the corresponding numerical values given beneath each piece of sclerenchyma are evidence of the much increased differentiation obtained by their use. These quantities allow eight varieties of cardamoms to be arranged in four distinct groups and, by supplementing the counts with other histological characters, the individual varieties can all be distinguished both in the unground condition and in the form of powder (Fairbairn²). This method deserves consideration wherever it is applicable and, in addition to the results for cardamom seeds, values already exist for the sclerenchymatous beaker-cell layer of pepper, viz. 1464 ± 200 beaker-cells per sq.mm. (Wallis and Santra³) and for the epidermal sclerenchyma of nux vomica seeds, viz. 570 epidermal sclereids per sq.mm. (Wallis and Fairbairn⁴).

SCLEREIDS PER MILLIGRAMME

Further advances in the use of sclerenchymatous tissues have been made by introducing the concept of mass into the values obtained. When this is done, it becomes possible, not only to identify the materials concerned, but also to assess the purity and to determine the proportions present in a given powder. Where the sclereids are isolated as in French savin, *Juniperus phœnicea* Linn., see Figure 1C (Flück and Haller⁵), or are loosely associated as in clove stalks, see Figure 1D (Wallis and

Santra⁶), and in the hypodermal sclerenchyma of pepper, see Figure 1B (Wallis and Santra⁷) and in the pericycle of ipecacuanha stem (Lupton⁸), the number of sclereids per mg. is easily counted. This is done by using the lycopodium method (Wallis⁹) and staining the material with phloroglucin and hydrochloric acid. The results for the materials quoted are as follows:—

Materials Examined.	Sclereids per mg.
French savin	210
Clove stalks	1,067
Pepper (hypodermal sclereids)	4,585
Black Pepper Husks (hypodermal sclereids)	13,230
Mysore Cardamom seeds (beaker-cells)	9,154
Black Pepper (beaker-cells)	1,500
White Pepper (beaker-cells)	1,619
White Pepper shells (beaker-cells)	10,783
Ipecacuanha stem	33

MEASUREMENT OF LENGTH PER MILLIGRAMME

Although the number of epidermal sclereids per mg. of *nux vomica* seeds has been determined by careful manipulation and found to be 288 per mg. (Wallis and Fairbairn⁴), this value cannot be used as a means of determining *nux vomica* in powder, because the trichomes obscure the outlines of the cell bases. Each epidermal sclereid is prolonged into a trichome, the limb of which is traversed longitudinally by several (average number 11) narrow lignified strips varying in width from 3 to 10 μ , see Figure 1E, e_1 and e_2 . In the powder of the seed the strips separate and become broken into small fragments, which have a very characteristic appearance and are easily recognised. It is preferable therefore and comparatively simple to determine the length per mg. of the fragments of lignified rib derived from the trichomes. For this purpose, the powdered *nux vomica* is mixed with lycopodium and stained with safranin and the lengths of the fragments of rib are measured by using a camera lucida. In this way, it is found that there are, on the average, 184 cm. of rib per mg. of air-dry *nux vomica* (Wallis and Fairbairn⁴).

All these values involving structural units per mg. can be used either to assess the purity of the material concerned or to determine the amount of any one of them in admixture with other substances.

MEASUREMENTS OF AREA

Fibres in vegetable materials are more difficult to count than sclereids; this is largely because of their length and the difficulty of deciding how many fibres are represented by the broken portions found in the powders. To obtain satisfactory results with powders of No. 85 fineness, work must, at present, be limited to those materials in which the fibres occur either isolated, as in sassafras bark, or are arranged in single rows, as in cinchona, cassia and cinnamon barks; fibres in *bundles cannot* be successfully counted in a No. 85 powder. The difficulty of counting the fragments present is best surmounted by finding the total area of the

SCLERENCHYMA IN DIAGNOSIS OF POWDERS

fragments of fibres present; this is done by tracing the outlines of the fragments with a camera lucida and finding their area by cutting out the tracings and weighing them. In conjunction with the lycopodium method, the area of fibre per g. is determined. This procedure was adopted for the fibres of powdered cinnamon and powdered cassia barks. The number of fibres in the phloem of cinnamon is considerably greater than in the phloem of cassia; moreover, the cork and cortex are removed from cinnamon, but not from cassia and this still further increases the difference between them. The fibres of cinnamon are somewhat more slender, but slightly longer than those of cassia, so that the area of the outlines of individual fibres in the two barks is not very different. The area of fibre-outline per mg. which summates number, length and breadth is therefore markedly greater in cinnamon than in cassia. The values obtained (Saber¹⁰) are:—

Cinnamon 80 to 85 to 91 sq.cm. per g.

Cassia 11 to 12 to 13 sq.cm. per g.

For these two barks therefore the values are widely separated and can be used, not only to characterise the barks themselves, but also to determine accurately the proportion of either in a mixture of the two or in compound powders.

Area measurements are also used for sclereids which form a layer one cell thick, as in linseed (see Fig. 1 F) (Saber¹¹) and in cardamom seeds, see Fig. 1 N, (Fairbairn¹), the result being expressed as area of the layer per mg. The values found for these seeds are:—

Linseed 28 to 32 to 35 sq. cm. per g.

Cardamom (Mysore) 27 to 28.5 to 29.7 sq. cm. per g.

These values can be used to determine the proportion of linseed in mixed cakes, etc., and of cardamom seeds in mixed spices and compound powders.

Many aerial stems are strengthened to withstand lateral strains by a tubular development of sclerenchyma, often in the pericycle, sometimes in the inner layers of the cortex. The stem bases attached to the rhizomes of *Valeriana officinalis* Linn., show a well-developed cylinder of rectangular sclereids in the inner layer of the cortex and there are similar cells in the bases of the petioles of the same plant. It has been proposed to use these diagnostic elements to determine the proportion of stem and leaf-bases present in powdered valerian rhizome (Flück and Haller²). The amount of these sclereids is measured in terms of their area obtained by multiplying together the length and breadth of the particles of sclereid layer seen in the powder. In this way 6.83 sq cm. of sclereid layer was found to be present in one gram of powdered stem and leaf base. Flück and Haller suggest that the Swiss Pharmacopœia might introduce a standard for powdered valerian rhizome of not more than 0.35 sq.cm. of these sclereids per g., corresponding to just over 5 per cent. of stems in the drug.

THREE-DIMENSIONAL MASSES OF CELLS

In many substances, sclereids are present in masses, which may be limited in extent and approximately ovoid in shape, as in pimento, or

Santra⁶), and in the hypodermal sclerenchyma of pepper, see Figure 1B (Wallis and Santra⁷) and in the pericycle of ipecacuanha stem (Lupton⁸), the number of sclereids per mg. is easily counted. This is done by using the lycopodium method (Wallis⁹) and staining the material with phloroglucin and hydrochloric acid. The results for the materials quoted are as follows:—

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SCLERENCHYMA IN DIAGNOSIS OF POWDERS

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THREE-DIMENSIONAL MASSES OF CELLS

In many substances, sclereids are present in masses, which may be limited in extent and approximately ovoid in shape, as in pimento, or

they may form a dense tissue, as in olive stones. These groups and tissues occur in the powder as three-dimensional particles and it is impossible to count accurately the individual cells in these particles. This difficulty can be overcome either by breaking down the particles into individual cells or by finding some method of calculating the number of cells from the number visible on the upper surfaces of the masses. For breaking down the particles, the oxidising agent used is nitric acid; an important drawback to this method is that the removal of lignin by the oxidising agent modifies or destroys the staining reactions of the cells, thus making it more difficult to identify them in the operation of counting. In attempting to devise a method for making calculations; it is evident that the shape of a mass built up of cells must depend to a large extent upon the shape of the constituent cells. If the cells are chiefly longer than wide, an ovoid mass might be expected, but if they are isodiametric, a subspherical mass would result. For both types of cell aggregate a count of cells along two axes at right angles would give a good estimate of the diameter of the sphere, when the mass is subspherical, or of an imaginary equivalent sphere, when the mass is more or less ovoid (see Fig. 3). Then using the formula for volume of a sphere, viz. $\frac{4}{3}\pi r^3$, the number of cells is calculated. If a sufficient number of particles is used—about 12 to 20—the result of the calculation method agrees with that obtained by disintegration and, since calculation is more rapid and involves no change in staining reaction of the cells, it is to be preferred. Proceeding in this way it has been found (Wallis and Santra¹²) that pimento contains 3546 (± 200) sclereids per mg.

Powdered olive stones consist of a certain number of individual cells

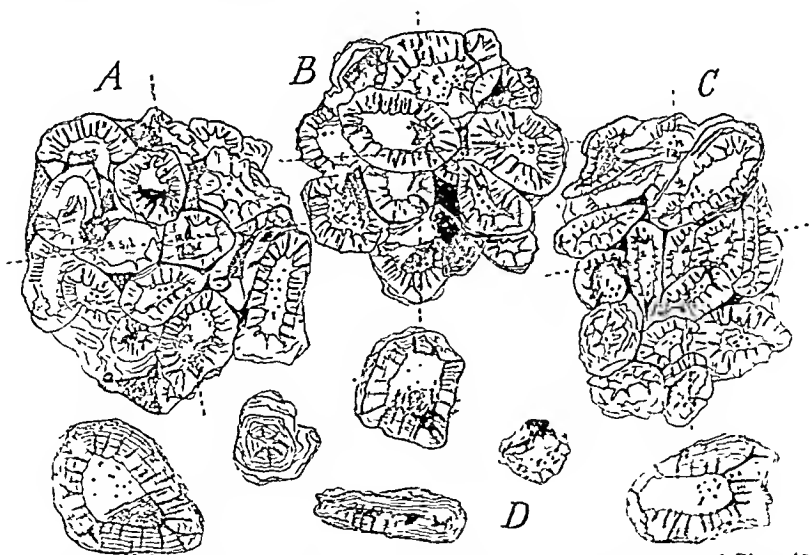


FIG. 3.—A, B, C, three typical masses of sclereids isolated from powdered *Pimento officinalis* Lindl.; D, individual sclereids of various shapes and sizes from powdered pimento. All $\times 200$. The dotted lines are the diameters across which the numbers of cells were counted in calculating the radii of the equivalent spheres.

and broken pieces as well as particles of various shapes consisting of masses of sclereids. The counting of the separated individual sclereids presents no special difficulty, but the particles consisting of masses of cells are much more irregular than the ovoid groups found in pimento. When, however, the same convention of an equivalent sphere is applied to them, it was found that, if at least 12 particles are used, the number of sclereids obtained by calculation agrees with the number found by disintegration. A standard value for olive stones was thus determined, viz:—15,140 (± 900) sclereids per mg. (Wallis and Santra¹). This value has been successfully applied for the determination of powdered olive stones added to pepper.

QUANTITATIVE DISTRIBUTION OF TISSUES

By utilising the number of sclereids per mg. of black and white pepper and of the various products (see above) obtained commercially in the grinding of pepper, it has been possible to obtain a quantitative measure of the distribution of the tissues in the fruit of *Piper nigrum* Linn. The values thus established can be used to assess the proportions of the different parts of the fruit, which should be present in commercial products obtained from peppercorns (Wallis and Santra³). The quantitative distribution of the tissues in black pepper berries and their products is as follows:—

Black pepper shell in pepper fruit	. . .	34.6 per cent.
White pepper shell in pepper fruit	. . .	13.9 per cent.
Perisperm in pepper fruit	51.5 per cent.
White pepper shell in white pepper	14.3 per cent.
Perisperm in white pepper (by difference)	85.7 per cent.

RELIABILITY OF THE NUMERICAL VALUES

Justification for accepting, as reliable and satisfactory, the general method of working by the use of lycopodium has been provided in connection with many of the experiments. This has been done by making a duplicate and independent determination of the result by a method which did not involve the use of lycopodium. For several commodities the required value can be found by the use of calculations based upon geometrical data derived from measurements of the area or volume or some other character of the unground substance. Whenever this has been done, working with sclereids it has been used for linseed, nux vomica and pepper, the values obtained have always been in remarkably close agreement with those found by the lycopodium method applied to the powder of the same material. Although this type of independent check cannot be made for every powder, the fact that it has confirmed values for a number of materials to which it is applicable gives justification for claiming a similar accuracy for all the materials examined. In this way complete confidence in the results has been established so that, when they happen to differ markedly from results obtained from powdered materials by other methods, the cause of disagreement must

be sought either in some defect inherent in the other method or in some difference in the actual substance examined.

When determining the number of cells per sq.mm. of cardamon seeds the results from the first series of experiments showed a rather large variation, which was greater than was desirable and trials showed that altering the details of manipulation yielded no improvement. It therefore appeared that the variations were probably due to variations in the number of pieces of sclerenchymatous layer used for each value obtained; these varied from 8 to 16 pieces selected at random from a powder of No. 85 fineness. Although a fairly precise estimate of the minimum number of pieces to be used could be made by considering the experimental figures obtained, it seemed desirable to obtain mathematical confirmation of the validity of the deductions made from the experiments. It was, therefore, determined to examine statistically the effect of using different numbers of pieces of the sclerenchyma. For this purpose the mean of the individual results from each of 98 pieces was found and the standard deviation was determined. Curves were then constructed to represent the limits of error above and below the mean that could be obtained by using gradually increasing numbers of pieces of the sclerenchyma. Two curves were constructed, one showing limits for errors in 67 per cent. of the counts and the other in 99 per cent. of the counts. In this way it was shown (Fairbairn¹⁴) that, when the number of pieces is about 36, the limits of error become fairly constant and for 99 per cent. of the determinations the limit of error is ± 8 per cent. and for 67 per cent. it is ± 3 per cent. This statistical examination creates confidence in the experimental figures based on counts of 36 pieces; it also gives a reliable measure of the amount of variation to be expected and therefore assists in attributing a correct degree of specificity to the values obtained.

CONCLUSION

A review of these studies of sclereids and fibres, made during the last 15 years, reveals the much extended information which can be gained by applying to them the concepts of number, length, area, volume and mass in addition to the simple observational concepts of form and location. Similar advances have been made in the study of other groups of tissues, but they cannot be discussed in this article.

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RESEARCH PAPERS

A TITRATION METHOD FOR THE DETERMINATION OF PROCAINE IN PROCAINE PENICILLIN AND ITS OILY SUSPENSIONS

By W. H. C. SHAW

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THE essential characteristics of procaine benzyl penicillin ("procaine penicillin") have been described by Salivar *et al*¹. It is a compound of one molecule each of procaine base and of benzyl penicillin (Penicillin G) with one molecule of water of crystallisation. Thus the theoretical composition is procaine 40.12 per cent., water 3.06 per cent. and penicillin potency 1008 I.U./mg., calculated on the basis of sodium benzyl penicillin as 1667 I.U./mg.

Procaine penicillin has been formulated for injection as a water-dispersible powder and also as a sterile suspension in arachis oil. Of these the latter is at present the more commonly used and normally contains 300,000 units of penicillin combined with 120 mg. of procaine base in each ml. This preparation may also contain 2 per cent. of aluminium monostearate as suspending agent². The high dosage and the toxicity of free procaine required the development of a rapid and accurate method for determining the procaine content of procaine penicillin both in the dry state and also when in oily suspension. Published methods for the determination of procaine include bromination, titration of the base with standard acid following either extraction with chloroform or separation by distillation³ and a colorimetric method for small amounts⁴. A spectrophotometric method for the determination of procaine in procaine penicillin G has recently been described⁵. It is well known, however, that most primary and secondary aromatic amines can be made to react quantitatively with nitrous acid. This method is used in the Pharmacopœial assay process for sulphanilamide and other sulphonamides but has apparently not been applied to procaine hydrochloride.

The object of the work described here was to investigate whether this reaction could be applied to the determination of procaine, particularly when in the forms already mentioned. The high cost of penicillin required that the amount used for each assay should be as small as possible and for this reason attention was directed to testing on a semi-micro scale.

EXPERIMENTAL

(1) *Titration of procaine hydrochloride.* Procaine hydrochloride B.P. was recrystallised twice from water and dried first at 60°C. and finally *in vacuo* over phosphorus pentoxide. Moisture content (Fischer reagent) 0.04 per cent., m.pt. 154.8° to 155.5°C. Found: C, 57.4; H, 7.72; N, 10.1%; ionisable chlorine, 13.00 per cent.

* I am indebted to Dr. F. R. Cropper for the results of all micro analyses quoted in this paper.

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$C_{13}H_{20}N_2O_2 \cdot HCl$ requires C, 57.23; H, 7.76; N, 10.27; ionisable chlorine, 12.99 per cent.

About 0.8 g. of the purified salt was dissolved in 15 ml. of 3N hydrochloric acid and the solution diluted to 150 ml. with distilled water. The solution was adjusted to 15°C. and titrated slowly (0.1 ml. at a time towards the end of the titration) with N/10 sodium nitrite solution. The end-point was taken when a drop of the titrated liquid gave an immediate blue colour on starch-iodide paper 2 minutes after the last addition of nitrite.

A blank determination omitting the procaine was carried out and the titre deducted. The results of a series of these titrations are given in Table I.

The determination was repeated on a semi-micro scale by dissolving about 0.1 g. of procain hydrochloride in 15 ml. of 3N hydrochloric acid and diluting to 50 ml. with water. After adjustment of temperature the solution was titrated as before with N/10 sodium nitrite added from a 5 ml. microburette in 0.02 ml. quantities towards the end of the titration. These results are also given in Table I.

TABLE I

TITRATION OF PURIFIED PROCAINE HYDROCHLORIDE WITH STANDARD SODIUM NITRITE SOLUTION

Procaine hydrochloride taken	N/10 sodium nitrite f 1.005	Procaine hydrochloride
g	ml	per cent
0.8009	29.22	99.93
0.8547	31.18	99.91
0.7525	27.44	99.88
0.8055	29.40	99.95
0.9033	32.93	99.86
0.1104	4.04	100.2
0.1238	4.51	99.8
0.1168	4.28	100.3
0.1218	4.45	100.0
0.1203	4.385	99.8

(II) *Titration of procaine penicillin.* (a) *Extraction of procaine from procaine hydrochloride.* When the above semi-micro procedure was applied to procaine penicillin fictitiously high results were obtained, apparently due to reaction of penicillin with nitrous acid. This was confirmed by titrating a sample of crystalline sodium penicillin in dilute hydrochloric acid solution with N/10 sodium nitrite. Slow absorption of nitrous acid occurred and no definite end-point was obtainable. Separation of procaine from penicillin was, therefore, necessary and an extraction technique was devised for this purpose. The procedure was first applied to procaine hydrochloride itself in order to check the efficiency of the extraction. The procaine hydrochloride used for this assayed 99.5 per cent. by the macro method given under (I).

About 100 mg. of procaine hydrochloride, accurately weighed, was dissolved in 50 ml. of water and the solution transferred to a separating funnel. 5 ml. of M ammonium hydroxide was added and the solution extracted successively with 20, 5, 5, and 5 ml. quantities of chloroform, previously washed by thorough shaking with an equal volume of

water.* Completion of extraction was checked by carrying out a fifth extraction with 5 ml. of chloroform, this being extracted with dilute hydrochloric acid and the acid solution tested for traces of procaine as described later. Each chloroform extract was run in turn into a 4 oz. wide-mouth glass stoppered bottle containing 35 ml. of water and 15 ml. of 3N hydrochloric acid. After adjusting the temperature to 15°C. the chloroform and acid layers were titrated with vigorous stirring with N/10 sodium nitrite solution in the manner described above until a reaction on starch-iodide paper was obtained. The stopper of the bottle was then inserted and the contents vigorously shaken for 30 seconds. After separation had occurred the titration of the aqueous layer was continued with stirring until an end-point reproducible after 2 minutes was obtained.

It was observed that any delay between addition of ammonia and extraction with chloroform led to low results. Decomposition of procaine in alkaline solution with the formation of *p*-aminobenzoic acid is well known⁶ and this was thought to be the cause of the low results. To test this and to show that the presence of penicillin did not interfere with the extraction the following three separate procedures were adopted:— (i) Extraction after addition of ammonia was delayed for a series of time intervals. (ii) 50 mg. of *p*-aminobenzoic acid was added before making alkaline. (iii) An approximately equimolecular proportion of crystalline sodium benzyl penicillin was added before making alkaline. The results obtained are given in Table II.

TABLE II

RESULTS OBTAINED USING THE EXTRACTION PROCEDURE ON PROCAINE HYDROCHLORIDE

Procaine hydrochloride	Crystalline sodium penicillin G added	Delay in extraction	N/10 sodium nitrite (f. 0.994)	Procaine hydrochloride (Mol. Wt. 272.6)
mg.	mg.	None	ml.	per cent.
117.4	—	None	4.31	99.5
120.4	—	"	4.44	99.9
122.5	—	"	4.43 (f 1.009)	99.4
127.5	151	"	4.61 (f 1.009)	99.4
123.7	158	"	4.545	99.6
122.1	150*	"	4.49	99.6
119.7	—	½ hr.	4.36	98.7
122.9	—	"	4.37 (f 1.009)	97.8
123.2	—	1 hr.	4.33 (f 1.009)	96.6
116.0	—	"	4.14	96.7
118.6	—	3 hrs.	3.99	91.1
115.1	—	"	3.86	90.9

* 50 mg. of *p*-aminobenzoic acid also included in this assay.

(b) *Extraction from procaine penicillin.* Direct application of the method used in (a) was inapplicable to procaine penicillin on account of its low solubility in water. About 280 mg. of procaine penicillin was, therefore, dissolved by warming in 15 ml. of chloroform contained in

* NOTE: In the presence of chloroform B.P. slow absorption of nitrous acid occurred in the blank titration but this could be prevented by prior washing of the chloroform with distilled water.

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a beaker and the solution poured into a separating funnel containing 50 ml. of water. A further 5 ml. of chloroform and 5 ml. of M ammonia were used in turn to rinse the beaker, each being added to the separating funnel. Extraction and titration procedure were as described above.

The sample* of procaine penicillin G used for this and replicate determinations assayed 1038 I.U./mg. (*Staphylococcus aureus*), 1016 I.U./mg. (*Bacillus subtilis*) water content (Fischer reagent) 3.4 per cent. Found: C, 59.15; H, 6.75 per cent.; N, 9.8; S, 5.31 per cent. $C_{29}H_{38}N_4O_6S \cdot H_2O$ requires C, 59.17; H, 6.85; N, 9.52; S, 5.45 per cent.

As a check on possible interference by low potency penicillin one assay where this was added is included in Table III.

TABLE III
REPLICATE DETERMINATIONS OF PROCAINE IN PROCAINE PENICILLIN

Weight taken	N/10 sodium nitrite f. 1.006	Procaine
mg.		per cent.
290.0	4.90	40.1
279.5	4.71	40.0
292.1	4.91	39.9
281.5	4.72	39.8
274.6	4.60	39.8
283.1	4.755	39.9
291.6	4.92	40.0
271.0	4.54	39.8
280.2	4.73	40.0
273.1	4.57	39.8
282.2	4.73	39.8
270.1	4.54	39.9
290.1*	4.86	39.8

* Sodium penicillin 0.1 g. (potency 547 I.U./mg.) added.

(III) Titration of Suspensions in Oil.

Accurate weights of the procaine penicillin used in (II) (b) were converted into suspensions in oil, using:—(i) 300,000 units in 1 ml. of arachis oil, (ii) as above, but with 2 per cent. of aluminium monostearate.

The procaine in these preparations was determined using the same technique as that previously adopted for procaine penicillin itself. The results are given in Table IV.

TABLE IV
DETERMINATION OF PROCAINE IN PROCAINE PENICILLIN WITH ADDED ARACHIS OIL AND ALUMINIUM MONOSTEARATE

Procaine penicillin taken	N/10 sodium nitrite f. 1.006	Procaine
mg.	ml.	per cent.
With arachis oil	4.89	40.0
290.5 ...	4.93	39.9
293.8 ...		
With arachis oil containing 2 per cent. w/w aluminium monostearate	4.78	39.9
284.1 ...	4.71	39.7
281.7 ...		

The same procedure was then applied to several routine manufactured batches of procaine penicillin suspension in arachis oil (with and without aluminium monostearate).

* I am indebted to Mr. D. H. Geard for this sample and to Mr. C. R. Bond for the results of all penicillin assays quoted.

These results are given in Table V.

TABLE V
REPLICATE DETERMINATIONS ON PROCAINE PENICILLIN OILY SUSPENSION
(300,000 I.U./ML.)

Sample	Density at 20° C.	Penicillin potency I.U./ml. (Iodimetric assay)	Replicate procaine determinations (calculated to Mol. Wt. 236.1)
A	0.994	295,000	per cent. w/v 12.0 12.0 12.0 12.0 12.0
B	0.996	303,000	12.2 12.1 12.2 12.1
C	1.001	309,100	12.6 12.6 12.6 12.4
D*	1.002	304,600	12.4 12.3 12.3 12.3

* Containing 2 per cent. w/w of aluminium monostearate.

CONCLUSIONS

Results given in Table I show that procaine may be determined satisfactorily under macro and semi-micro conditions by direct titration in acid solution with standard sodium nitrite.

By employing an extraction procedure, interference by penicillin may be eliminated with no loss of accuracy. The results in Table II show that extraction should follow immediately after addition of ammonia. otherwise, low results, due to decomposition of procaine, are obtained. For the same reason the chloroform extracts should be run directly into dilute acid.

The presence of chloroform in the final solution for titration in no way affects the results, but the emulsion formed by stirring or shaking during titration should be allowed to separate before removing a drop of the aqueous layer for spotting on to starch-iodide paper. In the presence of penicillin the first chloroform extract is normally turbid, but this has not been observed to affect the results. Vigorous shaking may produce emulsions that separate slowly and should, therefore, be avoided.

Although with a suitable microburette the volume of titrant used may be read to 0.002 ml., experience has shown that the end-point cannot be estimated to better than 0.02 ml., corresponding to an error of about 0.5 per cent. on a volume of 4 to 5 ml. This is borne out by the results quoted in Table III. Whilst this error would be correspondingly reduced by taking a larger sample for assay, it was considered tolerable for routine application.

It will be noted that starch-iodide papers have been used throughout

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place of the starch iodide paste recommended in the British Pharmacopœia for use in the assay of sulphanilamide. Papers have been found much more convenient, and they are reasonably stable if prepared and stored as described below.

RECOMMENDED METHOD

Special Reagents.

(a) *Chloroform.* Wash chloroform B.P. by shaking thoroughly with an equal volume of distilled water. Allow complete separation to take place and run off the chloroform for use.

(b) *Starch-iodide papers.* (i) Dissolve 4 g. of cadmium iodide in 50 ml. of warm water.

(ii) Make 5 g. of soluble starch into a thin smooth paste with a little distilled water. Pour the suspension into 450 ml. of boiling distilled water. Boil for 1 minute, add solution (i) and boil again for 1 minute. Cool the solution to 70° to 80°C. and impregnate strips of suitable filter paper (e.g., embossed filter paper No. 633 made by Evans, Adland and Co., Postlip Mills) by immersion in the solution and then removing as much surplus liquid as possible by means of a glass rod. Dry the impregnated filter paper in a warm atmosphere free from fumes. Cut off the edges of the strips and cut the remainder into strips for use. Store in a well-stoppered amber-coloured bottle.

Procedure.

Weigh accurately 0.27 to 0.29 g. of procaine penicillin (or about 0.9 g. of procaine penicillin suspension in oil, 300,000 I.U./ml.) and dissolve in 5 ml. of chloroform by warming. Pour the solution into a separating funnel containing 50 ml. of water. Rinse the beaker with 5 ml. of warm chloroform, then with 5 ml. of M ammonia, adding each in turn to the separator. Shake the contents of the separator gently for 2 minutes. Allow to separate, ignoring a turbidity of the chloroform layer and run the lower layer into a 4-oz. wide-mouthed bottle (provided with a well-fitting stopper) containing 35 ml. of water and 15 ml. of 3N hydrochloric acid.

Extract with 3 further portions each of 5 ml. of chloroform, running each in turn into the bottle. Adjust the temperature of the contents of the bottle to 15°C. and titrate with N/10 sodium nitrite with vigorous stirring until a drop of the aqueous portion of the titrated solution yields an immediate blue colour when spotted on starch-iodide paper. Insert the stopper of the bottle and shake vigorously for 30 seconds. Allow the layers to separate and complete the titration of the aqueous layer with gentle stirring. The end-point must be reproducible after allowing the titrated liquid to stand for two minutes after the last addition of nitrite, added 0.02 ml. at a time towards the end of the titration. Carry out a blank determination omitting the sample and deduct the titration figure. 1 ml. of N/10 sodium nitrite is equivalent to 0.02361 g. of procaine base.

NOTE 1: The extraction procedure described normally extracts the procaine quantitatively, the following test for traces of procaine may, however, be applied for confirmation.

Extract 5 ml. of chloroform solution with 4 ml. of water containing 0.2 ml. of N/10 hydrochloric acid. Allow to separate, discard the chloroform layer, warm the aqueous layer gently to expel chloroform, cool and add 1 ml. of N/10 iodine. No turbidity should be produced.

NOTE 2: Unless preparations of approximately known strength are being tested it is advisable to carry out a preliminary titration in order to establish the approximate end-point.

SUMMARY

1. A semi-micro method for the determination of procaine in procaine-penicillin and in its suspensions in arachis oil is given.

2. The method is based upon extraction of procaine base with chloroform followed by acidification of the extract and titration with sodium nitrite solution.

3. No interference has been encountered in the presence of low potency penicillin or aluminium monostearate.

The author wishes to thank Mr. A. G. Fishburn and Mr. R. T. Parry-Jones for their interest and helpful criticism during this work.

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A NEW METHOD FOR MEASURING DIFFUSION OF ANTISEPTICS FROM OINTMENT BASES

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ALTHOUGH it is not yet possible to determine by laboratory tests the clinical efficacy of medicated ointments, methods have been suggested for the measurement *in vitro* of certain physical properties which may influence their therapeutic action. For instance, it has frequently been found useful to study the rate of liberation of active agent from base under standard conditions. It is realised that data so obtained cannot be related directly to release of drug *in vivo* but it may, nevertheless, serve as a guide to the suitability of bases for specific purposes. The information may also provide a check on possible incompatibility between drug and excipients. Attention has so far been directed chiefly to the study of the diffusion of water-soluble drugs, usually antiseptics, from various bases and two general methods of measurement have been evolved:—(a) Measurement of zones of inhibition observed on inoculated agar following diffusion from a small cup¹; (b) Estimation by chemical or other suitable methods, of the amount of drug which diffuses into water through a cellophane or similar membrane^{2,3}.

Method (a) usually involves a minimum period of 24 hours contact between ointment and agar before any readings are made. It is thus not possible to compare different bases in respect of diffusion-rates over shorter periods of, say, 2 hours. There is also the drawback that it is not always easy to fill the cups completely and uniformly, as a result of which there may not be good agreement between duplicate experiments. In addition, the interpretation of results obtained by method (a) is very difficult, since not all antiseptics penetrate the agar gel in the same way. The antiseptic may—(1) diffuse in a simple manner through the agar gel, in which case the concentration of antiseptic falls steadily as the distance from the cup increases; (2) be weakly adsorbed on the agar, giving rise to a gradual fall in concentration of antiseptic from the cup outwards; the penetration of antiseptic is not, however, so extensive as in the first case; (3) be strongly adsorbed on the agar so that, from the cup outwards, there is a narrow zone of substantially constant antiseptic concentration followed by a rapid decrease to zero. Before conclusions can be drawn from any method involving transfusion into agar the precise nature of the process must be known. It is possible to compare different concentrations of the same antiseptic (e.g. penicillin) once the diffusion mechanism is known; it is very difficult to compare different antiseptics which may have dissimilar diffusion mechanisms.

Method (b) has the advantage of being adaptable to all water-soluble drugs, whether bacteriostatic or not, but requires a rather elaborate technique.

The object of the work described here was to devise a simple technique capable of detecting different rates of diffusion over shorter time intervals. It has resulted in the development of a test in which the active agent of an ointment diffuses through cellophane on to inoculated agar, and the minimum time of contact sufficient to cause inhibition of bacterial growth is recorded.

Attempts were made to devise a method of applying an ointment to inoculated agar in such a manner that it would be removable easily and completely after any desired time-interval. After a number of preliminary tests the procedure described below as Method I was selected as the most satisfactory and was thereafter evaluated by:—(a) Confirmation of reproducibility, (b) Comparison against other methods, (c) Utilisation to distinguish between bases of different type.

Method 1. 10 ml. of molten agar is poured into a petri dish and allowed to set. The plate is inverted in an incubator and dried for 2 hours. On to the layer of agar is poured 5 ml. of molten agar containing 1 ml. of a 1 in 10 broth dilution of a 24-hour broth culture of *Staphylococcus aureus*. Similar results are obtained if, instead of using two layers of agar, the culture is pipetted directly on to the surface of alcohol-dried agar, but it is difficult to ensure an even spread of the culture and it may take several hours for the liquid to become absorbed into the agar. The first method of inoculation was therefore considered preferable since it results in a dry, evenly-contaminated surface. By means of sterile forceps, four 1 inch squares of sterile cellophane are placed on the surface of the agar and incubated for 45 minutes. The incubation causes the cellophane to spread evenly on the agar. To absorb moisture a disc of filter paper is trapped in the lid of the dish. The preparation to be tested is applied to the surface of the cellophane by means of an all glass hypodermic syringe without needle, so as to leave a border about $\frac{1}{4}$ " wide on the cellophane. The plate is then returned to the incubator and pieces of cellophane with the adherent preparation under test, removed at suitable time-intervals. After incubation overnight the plates are examined for growth-inhibition which is indicated by a clear area of agar at the site of the cellophane square. The minimum time required to cause inhibition of growth is recorded. This period may vary from a

TABLE I

DIFFUSION OF ANTISEPTIC FROM PHENYLMERCURIC NITRATE JELLY 0.001 PER CENT. AS DETERMINED BY CELLOPHANE METHOD

Date Tested	Period of contact between treated cellophane and agar			
	1 min	5 mins	10 mins	15 mins
15.1.48	+	+	-	+
16.1.48	+	+	-	+
20.1.48	+	+	-	+
23.1.48	+	+	-	+
27.1.48	+	+	-	+

+ = complete clearance : = partial clearance : - = no clearance.

DIFFUSION OF ANTISEPTICS FROM OINTMENT BASES

few minutes to several hours and sighting experiments are therefore necessary when a preparation is tested for the first time.

Reproducibility. The test was applied to a range of active agents in a variety of bases (see Appendix) and no difficulty was experienced in obtaining replicate results. This is illustrated by Table I, which records successive tests made on a dilute phenylmercuric nitrate jelly.

Comparison with other methods. Although there may be objections to using variations of the agar cup method for evaluation of the diffusion of an antiseptic from ointment bases, there are no other methods available which are based on a biological technique. Accordingly, for comparison, it was decided to use three variations of the agar diffusion method, as detailed below.

Method 2. A cylindrical hole 15 mm. in diameter is cut in an agar plate inoculated as in Method 1, and a few drops of molten agar added, from a Pasteur pipette, to cover the glass surface so exposed. This hole is then filled with the preparation to be tested. The plate is incubated for 24 hours and the diameter of clearance measured.

Method 3. This method is similar to Method 2, but the preparation is placed in a ditch 10 mm. \times 40 mm. instead of in the cylindrical hole.

Method 4. Four sterile glass cylinders of internal diameter 10 mm. and depth 13 mm. are filled with the preparation to be tested and placed on the surface of a poured inoculated agar plate.

The results are recorded in Table II.

TABLE II
ANTISEPTIC ACTIVITY AS TESTED BY FOUR DIFFERENT METHODS

Base	Active agent	per cent	Method 1	Method 2	Method 3	Method 4
			Minimum time for inhibition minutes	Average diameter or clearance of 4 tests mm		
Jelly	{ crystal violet sodium ethyl mercurithio- salicylate	1 0 0 02	5	20	20	20
Jelly	sulphanilamide	5 0	5	20	18	10
Jelly	{ 4 4 - diamidinodiphenoxyprop- ane di-(hydroxyethanesulphonate)	0 15	10	4	4	4
Oil in-water emulsion	{ potassium hydroxyquinoline sulphate	0 2	15	20	18	10
	chlorocresol	0 2				
Petroleum lanolin	{ benzoyl peroxide chlorohydroxy quinoline	10 0 0 5	60	10	14	4
Simple ointment	phenylmercuric nitrate	0 001	60	7	6	4
Glycerin wool fat White soft paraffin	Resorcinol	12 5	hours 2	2	4	1
Oil in water cream	diamidinodiphenoxyprop- ane dihydrochloride	0 1	4	2	4	0

A number of preparations when tested in the same way gave negative results by all four methods, i.e., no diffusion of active agent could be detected. These included Scarlet Red Ointment B.P.C., Ichthamol Ointment B.P.C., Coal Tar Ointment B.P.C., and Salicylic Acid Ointment B.P.C. It will be noticed that a certain parallel exists between the results obtained by Method 1 and those from the remaining three inasmuch as increased times of contact usually correspond to decreased zones of inhibition, but Method 1 provides a much sharper distinction.

Distinction between different bases: Phenylmercuric nitrate and neutral proflavine sulphate were formulated into a series of five different types of base and at different concentrations. The results of applying Method 1 to these preparations are recorded in Table III.

TABLE III

MINIMUM TIME FOR INHIBITION OF BACTERIAL GROWTH BY VARIOUS OINTMENTS WHEN TESTED BY THE CELLOPHANE METHOD

Active ingredient				Fatty Base	Water-in-oil Base	Oil-in-water Base 1	Oil-in-water Base 2	Jelly
Phenylmercuric nitrate	per cent 0.001	60 mins.	10 mins.	15 mins.	15 mins.	2 mins.
"	0.002	30 mins.	10 mins.	4 mins.	4 mins.	3 mins.
Neutral proflavine sulphate	0.05	24 hrs.	24 hrs.	4 hrs.	45 mins.	30 mins.
"	0.1	24 hrs.	24 hrs.	4 hrs.	25 mins.	15 mins.
"	0.2	24 hrs.	24 hrs.	4 hrs.	15 mins.	5 mins.

The results obtained with this test seem to indicate that, other factors being equal, a jelly type of base is superior to oil-in-water emulsions, which in turn are better than water-in-oil emulsions or fatty bases. In fact, for water-soluble antiseptics, it would seem that the oil phase is largely redundant, its purpose being solely to increase the consistency of the preparation so that it will remain at the site of application. This function can be fulfilled equally well with the jelly base which has the added advantage of being cheaper and easier to prepare.

DISCUSSION

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DIFFUSION OF ANTISEPTICS FROM OINTMENT BASES

SUMMARY

1. A test is described for measuring the release of antiseptics from ointments.
2. The significance of the test is discussed and some applications suggested.

REFERENCES

1. Ruehle and Brewer, *U.S. Dept. Agric. Circ.*, No. 198, 1931.
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3. Fuller, Hawking and Partridge, *Quart. J. Pharm. Pharmacol.*, 1942, 15, 127.

APPENDIX

MATERIALS

Ointment Bases:

Fatty Base:	Simple Ointment B.P.	
Water-in-oil Base:	Hydrous Ointment B.P.	
Oil-in-water Base 1:	Active Agent	a sufficient quantity
	Emulsifying Wax B.P.	7 g.
	Hard Paraffin B.P.	5 g.
	Liquid Paraffin B.P.	40 g.
	Distilled Water, sufficient to produce	100 g.
Oil-in-water Base 2:	Active Agent	a sufficient quantity
	Castor Oil B.P.	20 g.
	Cetyl Alcohol	6 g.
	Diethyleneglycol distearate	9 g.
	Polyglyceryl ricinoleate	5 g.
	*Emulsifying Agent (non-ionic) ...	3 g.
	Distilled Water, sufficient to produce ..	100 g.

* A condensation product of cetyl alcohol with ethylene oxide.

Jelly:	Active Agent	a sufficient quantity
	Cellofas W.F.Z.	6 g.
	Distilled Water, sufficient to produce	100 g.
Cellophane:	Described as non-waterproof of 0.0009 inch thickness	
<i>Staphylococcus aureus</i> :	N.C.T.C. 4163	per cent.
Culture Medium:	Sodium Chloride	0.125
	Peptone	1.5
	Yeastrel	0.5
	Potassium dihydrogen phosphate (anhydrous)	0.1
	Dipotassium hydrogen phosphate (anhydrous)	0.1
	Dextrose	0.5
	Agar	2.0
	Distilled water (pH 7.2 to 7.6) to produce	100

A number of preparations when tested in the same way gave negative results by all four methods, i.e., no diffusion of active agent could be detected. These included Scarlet Red Ointment B.P.C., Ichthamol Ointment B.P.C., Coal Tar Ointment B.P.C., and Salicylic Acid Ointment B.P.C. It will be noticed that a certain parallel exists between the results obtained by Method 1 and those from the remaining three inasmuch as increased times of contact usually correspond to decreased zones of inhibition, but Method 1 provides a much sharper distinction.

Distinction between different bases: Phenylmercuric nitrate and neutral proflavine sulphate were formulated into a series of five different types of base and at different concentrations. The results of applying Method 1 to these preparations are recorded in Table III.

TABLE III

MINIMUM TIME FOR INHIBITION OF BACTERIAL GROWTH BY VARIOUS OINTMENTS WHEN TESTED BY THE CELLOPHANE METHOD

Active ingredient			Fatty Base	Water-in-oil Base	Oil-in-water Base 1	Oil-in-water Base 2	Jelly
Phenylmercuric nitrate	...	per cent					
	...	0.001	60 mins.	10 mins.	15 mins.	15 mins.	2 mins.
	...	0.002	30 mins.	10 mins.	4 mins.	4 mins.	3 mins.
Neutral proflavine sulphate	...	0.05	24 hrs.	24 hrs.	4 hrs.	45 mins.	30 mins.
"	"	0.1	24 hrs.	24 hrs.	4 hrs.	25 mins.	15 mins.
"	"	0.2	24 hrs.	24 hrs.	4 hrs.	15 mins.	5 mins.

The results obtained with this test seem to indicate that, other factors being equal, a jelly type of base is superior to oil-in-water emulsions, which in turn are better than water-in-oil emulsions or fatty bases. In fact, for water-soluble antiseptics, it would seem that the oil phase is largely redundant, its purpose being solely to increase the consistency of the preparation so that it will remain at the site of application. This function can be fulfilled equally well with the jelly base which has the added advantage of being cheaper and easier to prepare.

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	Cellofas W.F.Z.	6 g.
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Cellophane:	Described as non-waterproof of 0.0009 inch thickness	
<i>Staphylococcus aureus:</i>	N.C.T.C. 4163	
Culture Medium:	Sodium Chloride	per cent.
	Peptone	0.125
	Yeastrel	1.5
	Potassium dihydrogen phosphate (anhydrous)	0.5
	Dipotassium hydrogen phosphate (anhydrous)	0.1
	Dextrose	0.1
	Agar	0.5
	Distilled water (pH 7.2 to 7.6) to produce	2.0
		100

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Neutral proflavine sulphate	...	0.05	...	24 hrs.	24 hrs.	4 hrs.	45 mins.	30 mins.
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"	...	0.2	...	24 hrs.	24 hrs.	4 hrs.	15 mins.	5 mins.

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BROMINATION OF *p*-AMINOSALICYLIC ACID

The number of reactive positions R in the molecule of *p*-aminosalicylic acid, sodium *p*-aminosalicylate or *m*-aminophenol was calculated from the formula $R = \frac{EM}{50C}$ where E is the number of milli-equivalents of bromine absorbed, M is the molecular weight of the compound and C is the concentration of the test sample in g./l.

RESULTS AND DISCUSSION

The results of a series of titrations on the three materials is set out in Tables I, II and III. It appears that the materials examined brominate quantitatively under the conditions used and the reaction could be made the basis of an assay process.

TABLE I
THE BROMINATION OF *p*-AMINOSALICYLIC ACID
(ASSAY BY ALKALI TITRATION 99.74 PER CENT. W/W)
CONCENTRATION OF SOLUTION 1.200 G./L.

Bromine added milli-equivalents	Na ₂ S ₂ O ₃ required milli-equivalents	Bromine absorbed milli-equivalents	No. of reactive positions found	Percentage w/w of C ₆ H ₄ COOH.OH.NH ₂
1.500	330	1.170	2.99	99.5
2.000	822	1.178	3.01	100.2
2.000	828	1.172	2.99	99.7
2.500	1.320	1.180	3.01	100.3
2.500	1.323	1.177	3.00	100.1
2.500	1.323	1.177	3.00	100.1
2.500	1.325	1.175	3.00	99.9
2.500	1.324	1.176	3.00	100.0
2.500	1.328	1.172	2.99	99.7
3.000	1.828	1.172	2.99	99.7
3.500	2.330	1.170	2.99	99.5
3.500	2.326	1.174	2.99	99.8
4.000	2.822	1.178	3.01	100.2
5.000	3.820	1.180	3.01	100.3

TABLE II
THE BROMINATION OF SODIUM *p*-AMINOSALICYLATE DIHYDRATE
(CONTAINS 82.9 PER CENT. W/W OF C₆H₄COONa.OH.NH₂)
CONCENTRATION OF SOLUTION 1.600 G./L.

Bromine added milli-equivalents	Na ₂ S ₂ O ₃ required milli-equivalents	Bromine absorbed milli-equivalents	No. of reactive positions found	Percentage w/w of C ₆ H ₄ COONa.OH.NH ₂
1.500	365	1.135	2.48	82.8
2.000	866	1.134	2.48	82.7
2.500	1.360	1.140	2.49	83.2
2.500	1.365	1.135	2.48	82.8
3.000	1.865	1.135	2.48	82.8
3.500	2.360	1.140	2.49	83.2
4.000	2.860	1.140	2.49	83.2
5.000	3.867	1.133	2.48	82.6
5.000	3.866	1.134	2.48	82.7

* Theoretical figure equivalent to three bromine atoms when calculated for the anhydrous salt.

The results on *m*-aminophenol substantiate the results of Sprung¹ while the results on *p*-aminosalicylic acid and its sodium salt would appear to agree with Day and Taggart's² conclusions regarding a number of salicylates. The analyses suggest that three atoms of bromine are absorbed per molecule of the compounds studied. Although their point of reaction is a matter for conjecture it is interesting to note from Allen's Commercial Organic Analysis (4th Ed., 1910, Vol. III) that salicylic acid

THE BROMINATION OF *p*-AMINOSALICYLIC ACID, SODIUM *p*-AMINOSALICYLATE AND *m*-AMINOPHENOL

By D. SIMMONITE

From the Research Department, Heris. Pharmaceuticals Ltd.

Received March 29, 1949.

THE quantitative bromination of phenols by acid bromide/bromate solution, first described by Koppeschaar¹ in 1876 is based upon the general assumption that aqueous nascent bromine will substitute quantitatively only in the *ortho* and *para* positions of phenols. The presence of certain groups other than hydrogen in these positions has been found to give anomalous results. Ruderman² showed that certain alkylated phenols brominate quantitatively regardless of the bromine excess used while others overbrominate to an extent which varies with the magnitude of the bromine excess. Day and Taggart³ found that Koppeschaar's method was unsatisfactory for materials like *o*- and *p*-cresol but satisfactory for *m*-cresol, phenol and a number of salicylates. Sprung⁴ found that phenols substituted in the *meta* position brominate quantitatively. The present paper was undertaken to study the behaviour of *p*-aminosalicylic acid and *m*-aminophenol on bromination with the object of developing an assay method for these compounds.

EXPERIMENTAL

The *p*-aminosalicylic acid used was purified by two crystallisations from methyl alcohol. A melting-point is not quoted since the material is known to decompose on heating at temperatures over 110°C., the melting-point being variable according to the rate of heating⁵. The sodium *p*-aminosalicylate was purified by recrystallisation from hot 90 per cent. aqueous alcohol. The product contained two molecules of water of crystallisation. The *m*-aminophenol was recrystallised from boiling water; m.pt. 122° to 123°C.

Procedure.—An accurately weighed quantity of the material was dissolved in distilled water using sufficient sodium hydroxide in the case of *p*-aminosalicylic acid and *m*-aminophenol to effect solution and made up to 1 l. A 25 ml. aliquot of the solution was transferred to a 250 ml. iodine flask, to which were added varying volumes of N/10 potassium bromate/bromide solution, together with a volume of distilled water to ensure that the total volume of bromate/bromide solution and diluent water was 50 ml. 5 ml. of concentrated hydrochloric acid was then added, the flask was immediately stoppered and allowed to stand for 5 minutes, being shaken intermittently during this time. 5 ml. of a 20 per cent. w/v solution of potassium iodide was then added, the flask quickly stoppered and allowed to stand for a further 5 minutes. The stopper and sides of the flask were then washed down with water and the excess of iodine estimated by titration with N/10 sodium thiosulphate, using starch solution as indicator. A blank was run using an additional 25 ml. of distilled water in place of the test solution.

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brominates with loss of the carboxyl group and the formation of 2:4:6 tribromophenol.

From the present work it would appear that pure specimens of *p*-aminosalicylic acid and its sodium salt brominate quantitatively regardless of the excess of bromine used. It is interesting to notice, however,

TABLE III
THE BROMINATION OF *m*-AMINOPHENOL
CONCENTRATION OF SOLUTION 0.8000 G./L.

Bromine added milli-equivalents	Na ₂ S ₂ O ₃ required milli-equivalents	Bromine absorbed milli equivalents	No of reactive positions found	Percentage w/w of C ₆ H ₄ OH NH ₂
1.500	403	1.097	2.99	99.7
1.500	407	1.093	2.98	99.3
1.500	405	1.095	2.98	99.5
1.500	400	1.100	3.00	99.9
2.500	1.394	1.106	3.15	100.4
3.000	1.896	1.104	3.01	100.3
3.000	1.897	1.103	3.01	100.2
3.500	2.400	1.100	3.00	99.9
3.500	2.395	1.105	3.01	100.4
4.000	2.910	1.090	2.97	99.0
4.000	2.903	1.097	2.99	99.7
4.500	3.398	1.102	3.00	100.1
5.000	3.900	1.100	3.00	99.9
5.000	3.890	1.110	3.02	100.8
5.000	3.895	1.105	3.01	100.4

that as mentioned in an earlier communication⁵, less pure material shows a slight variation in bromine absorption which does depend upon the available bromine. Accordingly, a direct bromination method using an external indicator has been suggested for assay purposes, although the present method is more convenient where the degree of purity of the material warrants its application.

SUMMARY

1. The bromination of *p*-aminosalicylic acid, sodium *p*-aminosalicylate and *m*-aminophenol has been studied using a variable excess of bromide/bromate solution.

2. *p*-Aminosalicylic acid, sodium *p*-aminosalicylate and *m*-aminophenol brominate quantitatively regardless of the excess of bromide/bromate solution used.

3. The basis for an assay process for *p*-aminosalicylic acid, sodium *p*-aminosalicylate and *m*-aminophenol has been described.

Thanks are expressed to the Directors of Herts Pharmaceuticals Limited for permission to publish these results.

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A PHOTOELECTRIC COLORIMETRIC METHOD FOR THE ESTIMATION OF KHELLIN

By I. R. FAHMY, N. BADRAN AND M. F. MESSEID

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Received March 28, 1949

KHELLIN, $C_{14}H_{12}O_5$ (2-methyl-5:8-dimethoxy-6:7-furano-chromone^{1,2}) m.pt. 154° to $155^\circ C.$, the main active principle of the fruits of *Ammi Visnaga* Linn., is identified by the rose red colour which it gives with potassium or sodium hydroxide³. This test has been used as a spot reaction by Abdel-Rahman⁴ for the determination of khellin in solutions. It makes use of the fact that one drop of khellin solution gives with solid sodium hydroxide a rose red colour, only, if the quantity of khellin is not below a certain limit (1:505,000), which is the identification limit. Accordingly, 0.066 $\mu g.$ of khellin can be detected in one drop, assuming that 1 ml. of the solution equals 30 drops.

Applying this method quantitatively, a given test solution is progressively diluted with constant testing of drops taken from the various dilutions until the colour reaction fails. The concentration of khellin in the original solution can then be calculated from the dilution required to reach this limit.

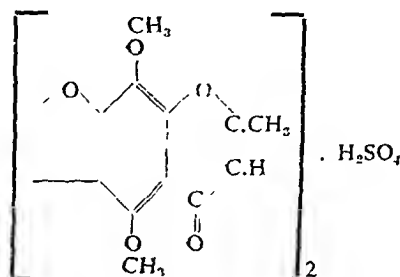
The repeated dilutions and testing of drops required by this procedure, are time consuming, and the results are only approximate, even if a special standardised dropper is used; this is because the "identification limit" is not the same for different observers; there is almost always a certain range of variation which depends on the individual characteristics of the observer. The range of dilution in which a test sometimes succeeds and sometimes fails is called by Feigl the "region of uncertain reaction⁵," and a quantitative estimation based on the examination for the failure of a test always extends into that region and therefore cannot be exact. Furthermore, even slight experimental errors become quite significant because the subsequent computation involves a multiplication. On applying this method, the results obtained by the authors varied to a great extent. In some determinations the results obtained were 40 to 60 per cent. lower than the amount of khellin taken.

The method of Anrep and co-workers⁶ for assaying khellin, using a saturated solution of potassium hydroxide, is a modification of the above method. Its advantage lies in the fact that it makes use of controls, the colour given by the unknown being matched with that of a standard solution; but a saturated solution of potassium hydroxide is not a stable reagent as it easily absorbs carbon dioxide from the atmosphere leading to precipitation of potassium carbonate, the result being a turbid solution which interferes with correct comparison of colours. By applying this method on a photoelectric colorimeter, the authors found that the transmission values of the coloured solution are different for the same amounts of khellin, when examined under the same experimental conditions. This shows that this method is not reliable for the quantitative

estimation of khellin. In this communication, a photoelectric colorimetric method is described. This method has the advantage of being accurate, simple and suitable for the assay of pharmaceutical preparations.

THE PHOTOELECTRIC COLORIMETRIC METHOD

Khellin gives with sulphuric acid a citron yellow colour which is stable in relatively dilute solutions. This colour is due to the formation of an oxonium salt (khellin sulphate) which has been isolated by the authors in a pure crystalline form as golden yellow needles having the structural formula.



The yellow colour of khellin sulphate is a property of the non-ionised salt. In presence of excess of water, khellin sulphate splits into khellin and sulphuric acid and the colour disappears as shown by the following equation:—



where K and K S represent khellin and khellin sulphate respectively.

This is a reversible reaction which follows the law of mass action. This equation in terms of concentration becomes:

$$\frac{(K S)}{(K) \cdot (H_2SO_4)} = \text{Constant.}$$

brackets being used to represent concentrations.

If the concentration of sulphuric acid or of khellin is increased; the concentration of khellin sulphate increases proportionally; and as khellin sulphate is responsible for the citron yellow colour produced; the optical density of the solution will be proportional to the concentration of khellin and of sulphuric acid present in the solution.

On keeping the concentration of sulphuric acid constant, the concentration of khellin sulphate and consequently the optical density of the solution will be proportional to the concentration of khellin.

$$\frac{(K S)}{(K)} = \text{constant or } \frac{D}{(K)} = \text{constant.}$$

where D represents optical density.

This is true within certain limits of concentration, as shown in the experimental part.

EXPERIMENTAL

Preparation of Khellin Sulphate. 10 g. of khellin is dissolved in 50 ml. of glacial acetic acid in the cold; then 25 ml. of concentrated sulphuric

ESTIMATION OF KHELLIN

acid is gradually added and the mixture is kept in the ice-chest for 24 hours. 150 ml. of absolute alcohol is then added slowly to the mixture, stirred and again kept in the ice-chest for 1 week. The golden yellow crystals of khellin sulphate which separate, are filtered by suction, washed twice with ethyl acetate, and dried at room temperature. Yield, 9.5 g. (95 per cent.); m.pt. 175° to 185°C.

The crystals when treated with water split into pure khellin, m.pt. 154° to 155°C., and sulphuric acid. Acid released, 15.635 per cent. w/w of H_2SO_4 . Khellin sulphate $\text{C}_{14}\text{H}_{12}\text{O}_5)_2 \cdot \text{H}_2\text{SO}_4$ requires 15.85 per cent. w/w.

RELATION BETWEEN THE CONCENTRATION OF KHELLIN, THE CONCENTRATION OF SULPHURIC ACID AND THE OPTICAL DENSITY OF THE SOLUTION

Solutions required:—

- (1) 10N sulphuric acid (A.R.).
- (2) Standard stock solution of khellin (0.5 per cent. w/v). 0.5 g. of pure crystalline khellin, m.pt. 154° to 155°C. dissolved in 100 ml. of alcohol (60 per cent. v/v).
- (3) Standard dilute solution of khellin (0.025 per cent. w/v); freshly prepared by diluting 5 ml. of the standard stock solution to 100 ml. with distilled water.

The relation between the concentrations of khellin and sulphuric acid and the optical density was determined at room temperature (25°C.).

The concentration of sulphuric acid in the solution is controlled by diluting known volumes of 10N sulphuric acid with distilled water to a constant volume, and the optical density of the solution is calculated from the equation:

$$D = 2 \log_{10} T$$

where D represents the optical density and T the percentage transmission of the solution. T is read directly on the scale of the colorimeter. Blue filter 420 is found to be the most suitable, as it gives minimum transmission values and a better spread of readings.

The following general procedure has been adopted. Into a volumetric flask of 50 ml. capacity, containing x ml. of a standard solution of khellin, n ml. of 10N sulphuric acid is added. The volume is then completed to 50 ml. with distilled water, well mixed, and left to stand for about 5 minutes; about 10 ml. of the solution is transferred to a colorimeter tube; and its percentage transmission is read in a Lumetron Photoelectric colorimeter Model 400-A using blue filter 420 against water as the blank set at 100 per cent. transmission, $x = 1$ ml. of the standard stock solution of khellin (0.5 per cent. w/v), representing a concentration of 10 mg. per cent. w/v of khellin, or 1, 2, 4, 8 and 10 ml. of the standard dilute solution of khellin (0.025 per cent. w/v) representing concentrations of 0.5, 1, 2, 4 and 5 mg. per cent. w/v of khellin respectively; $n = 10, 15, 20, 25, 30$ and 40 ml. of 10 N sulphuric acid, representing concentrations of 98.08, 14.712, 19.616, 24.52, 29.424 and 39.232 g. per cent. w/v of sulphuric acid respectively.

From the results obtained it may be concluded:—

(a) For the same concentration of khellin; the optical density of the solution increases with an increase in the concentration of sulphuric acid.

(b) For the same concentration of sulphuric acid the optical density of the solution increases with an increase in the concentration of khellin.

(c) For concentrations up to 4 mg. per cent. w/v of khellin the optical density is proportional to the concentration of khellin provided that the concentration of the acid is more than 29.424 per cent. w/v of sulphuric acid.

(d) Transmission readings obtained with a concentration of 39.232 per cent. w/v of sulphuric acid are spread enough to allow a determination of khellin to be done within concentrations ranging from 0.5 to 10 mg. per cent. w/v of khellin.

(e) The graph shows that within the above concentrations (0.5 to 10 mg. per cent. w/v of khellin) there is a slight deviation from Beer's Law. Therefore, a calibration table will replace more conveniently the graph and give more accurate results, when the calibration table and the estimations are made at the same room temperature and under the same conditions.

CALIBRATION TABLE

As a concentration of 39.232 per cent. w/v of sulphuric acid may be practically obtained by mixing 10 ml. of 10N sulphuric acid with 2.5 ml. of aqueous khellin solution the following procedure has been adopted for the preparation of the calibration table.

From the standard stock solution of khellin (0.5 per cent. w/v) are prepared standard dilutions so that 2.5 ml. of each dilution contain an amount of khellin ranging from 0.05 to 1 mg. and increasing in the order of 0.05 mg.; 2.5 ml. of each dilution are accurately measured in a dry colorimeter tube; 10 ml. of 10N sulphuric acid are added, well mixed; left to stand for about five minutes, then the percentage transmission of the solution is read, in a Lumetron Photoelectric colorimeter using blue filter 420 against water as the blank set at 100 per cent. transmission.

The results obtained at room temperature (25°C.) are shown in Table I.

TABLE I
CALIBRATION TABLE

Concentration of Khellin in mg. per cent.	Amount of Khellin in mg.	Percentage Transmission	Concentration of Khellin in mg. per cent.	Amount of Khellin in mg.	Percentage Transmission
0.4	0.05	93.0	4.4	0.55	49.0
0.8	0.10	87.0	4.8	0.60	47.0
1.2	0.15	80.5	5.2	0.65	44.0
1.6	0.20	75.5	5.6	0.70	41.0
2.0	0.25	71.0	6.0	0.75	39.0
2.4	0.30	65.0	6.4	0.80	37.5
2.8	0.35	61.0	6.8	0.85	35.0
3.2	0.40	57.0	7.2	0.90	33.0
3.6	0.45	54.0	7.6	0.95	31.5
4.0	0.50	51.0	8.0	1.00	30.0

ESTIMATION OF KHELLIN

METHOD OF ASSAY

(1) For the estimation of khellin in a test solution the following method is recommended. Make a dilution of the test solution so that 2.5 ml. contain an amount of khellin ranging between 0.05 and 1 mg. Accurately measure 2.5 ml. of this dilution into a dry colorimeter tube, add 10 ml. of 10N sulphuric acid; mix well; leave to stand for about 5 minutes; measure the percentage transmission of the solution and read the amount of khellin corresponding to the percentage transmission from the calibration table. The amount of khellin in the original test solution can then be obtained by calculation.

(2) For the estimation of khellin in oily preparations the following method is recommended. Dilute a known volume of the oily preparation with light petroleum so that 10 ml. of this dilution contains an amount of khellin ranging between 1 and 8 mg. Accurately measure 10 ml. of this dilution in a dry separating funnel; add 100 ml. of 39.232 per cent. w/v of sulphuric acid (obtained by diluting 80 ml. of 10N sulphuric acid to 100 ml. with distilled water); shake the mixture for about 15 minutes, then leave to stand for 15 minutes to separate. Filter about 15 ml. of the aqueous layer into a dry colorimeter tube; measure the percentage transmission of the solution and read the concentration of khellin corresponding to the percentage transmission from the calibration table. The figure obtained is the amount in mg. of khellin contained in 10 ml. of the diluted oil solution.

DISCUSSION

It has been found experimentally that, on mixing at room temperature 2.5 ml. of an aqueous solution of khellin with 10 ml. of 10N sulphuric acid the optical density of the solution becomes stable after leaving the mixture to stand for about 5 minutes at room temperature; and it remains stable for more than 24 hours.

On mixing the solution of sulphuric acid with the solution of khellin a rise of about 2°C., in the temperature and a contraction in the volume of about 0.5 ml. per cent. of the solution takes place. The rise in temperature and the contraction in the volume of the solution within these limits do not affect, to any appreciable extent, the optical density of the solution, i.e., a difference of about 2°C. in the temperature and a variation in the volume up to 0.5 ml. per cent. of the solution does not affect the optical density of the solution to any appreciable extent. On applying this method to accurately weighed amounts of pure khellin the results obtained did not differ by more than ± 2 per cent.

Visnagin, another constituent of the fruits of *Ammi Visnaga* Linn., may be present, as an impurity, in pharmaceutical preparations of khellin to an extent of about 5 to 10 per cent. of the weight of khellin present. As visnagin gives with sulphuric acid a yellow colour which, when compared with that given by khellin, is found to be about 50 per cent. less in intensity. In this case, the results obtained will not differ by more than ± 5 per cent.

Moreover, alcohol was found to interfere to a certain extent with the proper development of the colour. Therefore, alcoholic solutions of

khellin should be diluted with water before applying this method, so that the alcohol content of the coloured solution does not exceed 1 per cent, v/v.

SUMMARY

(1) A photoelectric colorimetric method for the assay of khellin is described.

(2) This method is recommended for the assay of pure khellin in pharmaceutical preparations.

(3) The assay is carried out within the limits of 0.1 to 1 mg. of khellin.

Work is proceeding for the application of this method on galenical and other pharmaceutical preparations. The results will be compared with those obtained by the other colorimetric methods.

The authors wish to express their thanks to the Memphis Chemical Co. for the help extended to them in carrying out this work and for the supply of pure khellin and instruments involved.

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KHELLIN AND ITS ASSAY IN INJECTIONS AND TABLETS

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THE most important constituent of *Ammi Visnaga* fruits, as regards pharmacological activity, is khellin, $C_{14}H_{12}O_5$, m.pt. 153° to 155° . Khellin is a furano-chromone derivative with a pronounced antispasmodic action on smooth muscle viz.: ureter, bronchial muscle, intestine and biliary duct^{1,2}. It has, moreover, a specific dilator action on the coronary arteries which makes it useful in the treatment of angina pectoris².

Besides khellin, the fruits contain two other constituents which have been isolated in chemically pure form and their molecular and structural formulæ established viz.: a glucoside, khellol-glucoside $C_{13}H_{10}O_5 \cdot C_6H_{11}O_5 \cdot 2H_2O$, m.pt. $175^\circ C.$ and visnagin⁴, $C_{13}H_{10}O_4$, m.pt. 142° to $145^\circ C.$, both of which are also furano-chromone derivatives. The glucoside is devoid of the antispasmodic action of khellin while visnagin has a much lower activity than khellin². The fruits contain about 1 per cent. of pure khellin, 0.1 per cent of pure visnagin and 0.3 per cent. of pure khellol-glucoside.

Khellin is usually obtained from the powdered fruits by extraction with ether or light petroleum, concentrating the extract and leaving it to crystallise; or alternatively the powdered fruits are extracted with alcohol, the alcohol is distilled and the residue extracted with chloroform. After distillation of the chloroform, crude khellin is obtained. By both methods, khellin is extracted in association with visnagin; the glucoside being insoluble in ether, in light petroleum and in chloroform. Khellin must be purified by several crystallisations from alcohol to free it from impurities and from visnagin. Nowadays, a number of pharmaceutical laboratories in Egypt prepare khellin on a semi-large scale. It is generally dispensed in the form of tablets or injectable solutions.

Up to the present, no standard description of khellin has been given. In this communication, the description, solubilities, identification, physical test of khellin and the method of its assay in the above preparations are described.

Description.—Khellin (2-methyl-5:8-dimethoxy-6:7-furano-chromone, Mol. Wt. 260), occurs in colourless needle-shaped crystals, odourless, slightly bitter. It should contain not less than 99 per cent. of $C_{14}H_{12}O_5$.

Solubilities.—Khellin is very soluble in chloroform, less soluble in ether and in light petroleum, more soluble in the hot liquids. Solubility: 25°C. in 130 parts of alcohol (95 per cent.) in 6750 parts of water in 500 parts of a saturated aqueous solution of theophylline, and in 33 parts of a saturated solution of sodium benzoate. It is soluble in glacial acetic acid and in dilute mineral acids from which it is unchanged.

Identification Tests.—(a) When one drop of 0.01 per cent w/v solution

khellin should be diluted with water before applying this method, so that the alcohol content of the coloured solution does not exceed 1 per cent. v/v.

SUMMARY

(1) A photoelectric colorimetric method for the assay of khellin is described.

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(3) The assay is carried out within the limits of 0.1 to 1 mg. of khellin.

Work is proceeding for the application of this method on galenical and other pharmaceutical preparations. The results will be compared with those obtained by the other colorimetric methods.

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Besides khellin, the fruits contain two other constituents which have been isolated in chemically pure form and their molecular and structural formulæ established viz.: a glucoside, khellol-glucoside³, $C_{15}H_{16}O_5 \cdot C_6H_{12}O_5 \cdot 2H_2O$, m.pt. $175^\circ C$. and visnagin⁴, $C_{13}H_{10}O_4$, m.pt. 142° to $145^\circ C$., both of which are also furano-chromone derivatives. The glucoside is devoid of the antispasmodic action of khellin while visnagin has a much lower activity than khellin². The fruits contain about 1 per cent. of pure khellin, 0.1 per cent of pure visnagin and 0.3 per cent. of pure khellol-glucoside.

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Solubilities.—Khellin is very soluble in chloroform, less soluble in cold ether and in light petroleum, more soluble in the hot liquids. Soluble at $25^\circ C$. in 130 parts of alcohol (95 per cent.) in 6750 parts of water⁵, in 500 parts of a saturated aqueous solution of theophylline, and in 33 parts of a saturated solution of sodium benzoate. It is soluble in glacial acetic acid and in dilute mineral acids from which it is regained unchanged.

Identification Tests.—(a) When one drop of 0.01 per cent w/v solution

in alcohol or in water is added to a piece of solid sodium or potassium hydroxide, a rose red colour is developed within 2 minutes^{7,8}.

(b) When a few crystals are treated with 1 drop of concentrated sulphuric acid on a white porcelain plate, a deep orange colour is developed which on dilution with water becomes yellow⁸.

(c) When a solution of 10 mg. in 2 ml. of alcohol (50 per cent.) is poured on a freshly prepared mixture of 0.5 ml. of N/2 iodine and 0.5 ml. of 10N potassium hydroxide solution, a yellow colour is formed followed by a yellow precipitate, which redissolves gradually on shaking, imparting to the solution a wine red colour.

Test for Purity.—m.pt. 153° to 155°C.

INJECTION OF KHELLIN

In consequence of the greater solubility in water in presence of theophylline or sodium benzoate, solutions for injection in an aqueous medium containing one or both of these compounds are already to be found on the Egyptian market.

Identification and Test for Purity.—Place in a separating funnel a volume of the injection equivalent to about 0.2 g. of khellin. Extract the khellin from this solution with three successive quantities, each of 10 ml. of pure benzene. Evaporate the combined benzene extracts to dryness on a water-bath and dry the residue at 100°C. The residue should comply with the tests for khellin.

Assay.—In solutions for injection, khellin may be assayed colorimetrically by the sulphuric acid method⁶. Dilute a volume of the solution equivalent to about 0.1 g. of khellin with distilled water to 100 ml. Dilute 10 ml. of this dilution to 50 ml. Measure 2.5 of this dilution in a dry colorimeter tube, add 10 ml. of 10 N sulphuric acid, leave to stand for about 5 minutes and read the percentage transmission in a photoelectric colorimeter against water as the blank, set at 100 per cent. transmission using blue filter No. 420. Read the amount of khellin corresponding to the percentage transmission from a calibration table, prepared under the same conditions, using standard dilutions of pure khellin. The result obtained, multiplied by 200, gives the amount of khellin present in the original volume taken.

TABLETS OF KHELLIN

Identification and Test for Purity.—Triturate a quantity of the powdered tablets, equivalent to 0.2 g. of khellin, with two successive quantities, each of 10 ml., of chloroform. Filter, evaporate the chloroform to dryness, on a water-bath, and dry the residue at 100°C. The residue should comply with the tests for khellin.

Assay.—Weigh and powder 20 tablets; treat an accurately weighed quantity of the powder, equivalent to about 0.1 g. of khellin, on a dry filter with successive small quantities of hot alcohol (95 per cent.) until the khellin is completely extracted. Concentrate the alcoholic extract to about 15 ml. and transfer it to a volumetric flask of 100 ml. capacity, washing the flask with two successive quantities, each of 5 ml., of alcohol

KHELLIN IN INJECTIONS AND TABLETS

(95 per cent.) and make up to volume with distilled water. Dilute 10 ml. of this solution to 50 ml. with distilled water. Carry out the assay, using 2.5 ml. of this dilution, as described for injections. The result obtained, multiplied by 200, gives the amount of khellin in the original weight taken.

DISCUSSION

The determination of the melting point of the extracted khellin from injections and tablets is required as a test for purity. If the khellin used is contaminated with visnagin or with other impurities from the fruits, the product will begin to melt below 140°C. In such cases the result of the assay may be reported as "total chromones of *Ammi Visnaga* fruits calculated as khellin."

In the extraction of khellin from injections, benzene is used instead of chloroform, to avoid the extraction of any theophylline present, theophylline being insoluble in benzene. Theophylline and sodium benzoate do not interfere with the method of assay.

SUMMARY

- (1) The characters and identification tests of khellin are described.
- (2) The standards of purity of khellin used in pharmaceutical preparations is given.
- (3) The method of assay of khellin in injections and tablets is described.

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THE RESPONSE OF THE HEART TO VISAMMIN AND TO KHELLININ

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INTRODUCTION

It has already been reported that various active principles are present in the fruit of *Ammi Visnaga* Linn, with different pharmacological actions and therapeutic indications^{1,2,3}. At the time of isolation of these principles in 1930 in this Department, the literature found on the chemistry of this drug was by Ibrahim Mostapha⁴ and T. Malosse⁵. The latter described three crystalline principles which he named visnagine α , visnagine β , and visnagine γ . Ibrahim Mostapha described a crystalline principle which he named khellin, and which was stated by Malosse (*loc. cit.*) not to be identical with visnagine α , but was supposed to be formed during the process of extraction. In view of this and other anomalies (variation of m.pt., etc.) it was deemed preferable to give new names to the isolated compounds, names which are derived from the Latin and Arabic names of the drug—*Ammi Visnaga* and *Khella* respectively.

Two compounds of the various principles isolated, namely visammin and the glycoside khellinin, are of great interest pharmacologically.

Anrep *et al.*^{6,7,8}, who recently worked on the active principles of *Ammi Visnaga*, refer to khellinin as khellol glycosides regardless of the fact that khellinin is not an alcohol, and that names of glycosides end in "in." The same authors refer to visammin as khellin.

The value of *Ammi Visnaga* as an antispasmodic and as a coronary dilator was indicated by one of us as early as 1930⁹. In what concerns the action of visammin and of khellinin on the circulation, experimental animals being the toad, rabbit and dog, the following points were indicated. (a) Visammin¹ in a concentration of 1:100,000 diminishes the amplitude of beat and slows the heart with no arrest. Stronger solutions (1:50,000 to 1:20,000) produce more evident slowing and a great diminution of the amplitude of beat. The diminished amplitude is relatively much more marked than the slowing, and is, to a great extent, due to diminution of systole rather than of diastole. Atropine added to the perfusion fluid produces no evident changes in the amplitude. The action is considered to be a direct depressant effect on the muscle fibres. Visammin is a systemic and coronary vasodilator. The intravenous injection of visammin produces an immediate fall of blood-pressure which returns to normal. Later it falls slightly below the normal and remains so for a long time. The first fall of blood-pressure occurs before and after atropine, but is more marked when no atropine is injected before injecting the drug. Similarly the fall of blood-pressure is more marked when the vagi are intact than when severed. Pre-

sumably, therefore, the initial fall of blood-pressure is mainly cardiac in origin and is due to the direct action of visammin on cardiac muscle and to cardiac inhibition through stimulation of the vagal centre. The intestinal volume shows an early slight decrease which may be explained as a secondary effect to the immediate action of the drug on the heart. Soon, the splanchnic vessels dilate as evidenced by the increase in intestinal volume. The vasomotor centre appears to be stimulated as indicated by the return of blood-pressure to normal after the first fall with a secondary diminution in intestinal volume, while, lastly, the direct action of visammin on the muscle wall of the blood vessels overshadows the stimulant effect on the vasomotor centre and results in a final slight fall of blood-pressure with increase in intestinal volume. (b) Khellinin^{1,3} in a concentration of 1:100,000 increases the contractility of the cardiac muscle by producing a more complete systole and a more complete diastole with a corresponding increase in cardiac output. It increases the coronary flow, and this increase is more pronounced if the coronary vessels were first rendered in a state of partial spasm by barium chloride (1:40,000). The intravenous injection of the glycoside with the vagi intact slightly raises the blood-pressure with an increase in intestinal volume. The rise in blood-pressure is cardiac in origin.

Recently Anrep *et al.*^{6,7,8} and Kenawy and Barsoum^{10,11} state that visammin is not a cardiac depressant, and that the administration in a heart-lung preparation of a dog of 100 mg. of the drug produces no change in the heart volume. The same authors record that the glycoside khellinin is not a cardiac stimulant and does not increase the coronary flow, and is devoid completely of pharmacological activity. Nevertheless, Bagouri¹², just recently, has admitted in his experiments that visammin causes a diminution in the amplitude of the heart beat which is of a temporary nature, and that khellinin produces a slight increase in the strength of heart beat with large doses of the glycoside.

This being the case, we thought it worth while to record the following experimental results and observations, in view of the fact that we have examined both principles in their crude forms and in their various stages of purity obtained during the processes of their final isolation—results and observations which may throw light on these variations of results.

EXPERIMENTAL

(A) *Intact animal*: The dog was used in all experiments. Anaesthesia was maintained by the intravenous injection of 0.22 g. of barbitone sodium per kg. supplemented by ether for the preliminary operative procedure. Blood-pressure was measured from the femoral artery. Injections were made in the femoral vein of the opposite side. In those experiments in which the ventricular beats were recorded, uniform artificial respiration was maintained by the use of Brodie's ideal respiration pump. The thorax was opened in the usual way. The pericardium was snipped through and ligatured to the anterior thoracic wall on each side.

A fine hook was applied to the tip of the left ventricle and connected to a recording lever. In some experiments the vagi were severed. We restricted ourselves to the injection of limited doses of visammin and of khellinin so as not to interfere with blood volume—the solubility of these principles in Ringer's solution being, indeed, very limited—and we avoided the interference of solvents (alcohol or sodium benzoate for visammin and alcohol or pyridine for khellinin) and the use of controls.

The injection of 3 to 4 mg. of pure visammin (m.pt. 153° to 154°C.) per kg. reduced the contractility of the ventricular muscle, producing principally a less complete systole. The diminution of the amplitude of beat was always evident, but was more marked when the vagi were intact (Fig. 1), and slowing of the rate of beat may later occur. The first

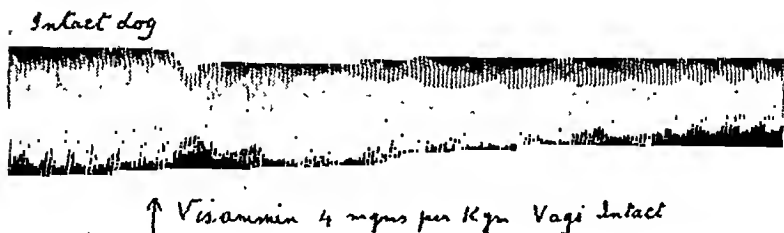


FIG. 1 (reduced).—Intact dog under barbitone sodium and uniform artificial respiration. The ventricular heart beats are recorded with the vagi intact. Intravenous injection of 4 mg. of visammin per kg. reduced the contractility of the ventricular muscle producing principally a less complete systole.

effect on blood-pressure was an initial fall followed by recovery with fluctuation—as previously reported the direct depressant action of visammin on the heart and the vasodilatation by its direct action on the muscle wall of the blood vessels contribute to the lowering of blood-pressure, whereas the stimulant action of the drug on the vasomotor centre may overshadow this effect.

On the other hand, the injection of 1 to 1.5 mg. of pure khellinin (m.pt. 175° to 176°C.) per kg, increased the contractility of the ventricular muscle producing a more complete systole and a more complete diastole. The increase in amplitude of the ventricular beats was always evident, but was more marked when the vagi were severed (Fig. 2), the rate of beat hardly changed though, relatively, slowing of the heart beat may occur with the vagi intact. The glycoside in similar doses raised the blood pressure in the intact animal especially when the vagi were severed (Fig. 3). This effect is, however, in favour of increased cardiac output—the glycoside does not directly stimulate the vasomotor centre and has no

RESPONSE OF HEART TO VISAMMIN AND KHELLININ

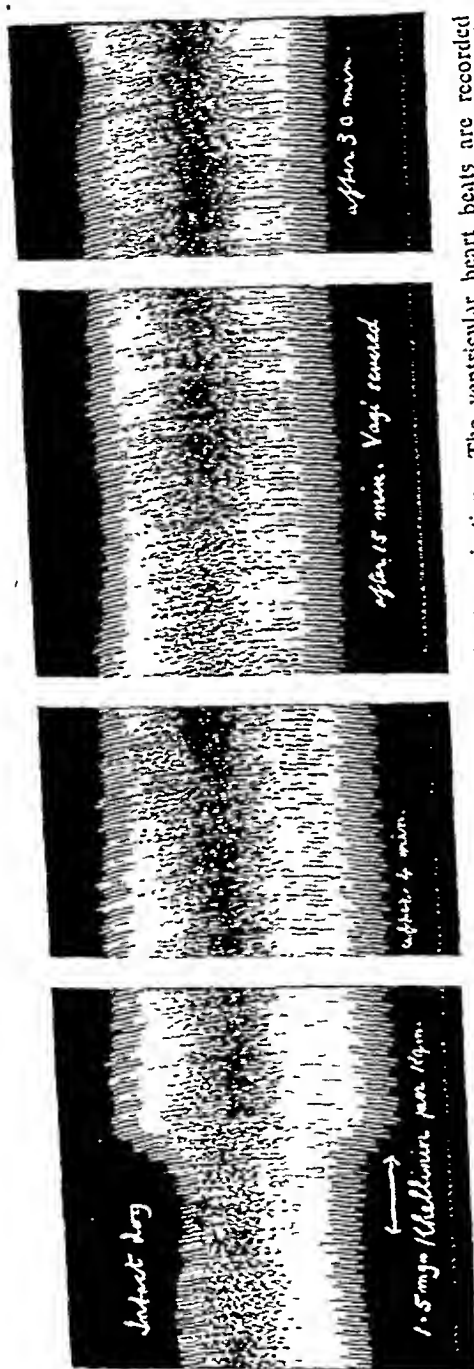


FIG. 2 (reduced).—Intact dog under barbitone sodium and uniform artificial respiration. The ventricular heart beats are recorded with the vagi severed. Intravenous injection of 1.5 mg. of khellinin per kg. increased the contractility of the ventricular muscle producing a more complete systole and a more complete diastole. The rate of beat hardly changed. Record after 4, 15 and 30 minutes of injection is demonstrated.

direct vasoconstricting action on blood vessels. The stimulant action of khellinin on the heart is well exhibited in such small doses and is persistent and rather "selective"—the glycoside, as already described by one of us, has no appreciable action on other organs in even larger doses.

(B) *Toad's heart perfusion*: Several experiments were carried out with various concentrations of visammin (1:10,000 to 1:100,000) and of khellinin (1:20,000 to 1:200,000). All the results were depression with visammin and stimulation with khellinin, with no cumulation. We record (Fig. 4) a triple perfusion, first with Ringer's solution, then with visammin (1:35,000 in Ringer's solution) and lastly with the same solution of visammin in which khellinin is dissolved in the same concentration. Depression under visammin is noticeable and the stimulant effect of khellinin is illustrated. In fact this antagonism was referred to by one of us as early as 1932 when it was recorded. "The cardiac depression caused by solutions of visammin is much more marked than equivalent concentrations of tinc-

ture of Ammi Visnaga —the presence of khellinin in the tincture partly accounts for this."

Moreover, Figure 4 demonstrates a greater amplitude of heart beat with visammin and khellinin together than under normal Ringer's solu-

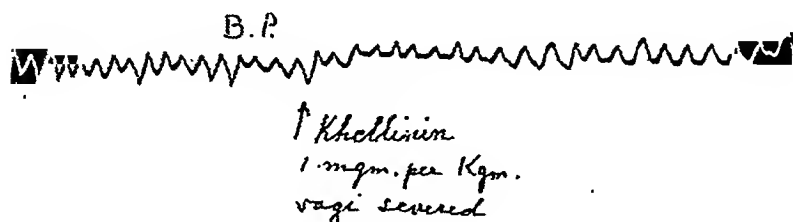


FIG. 3 (reduced).—Blood pressure of a dog under barbitone sodium. Vagi severed. Intravenous injection of 1 mg. of khellinin per kg. raised the blood-pressure.

tion. Presumably, however, one may infer that in the same concentration khellin is more a cardiac stimulant than visammin is a cardiac depressant. The perfusion of a strong solution (1:10,000) of visammin (Fig. 5) greatly diminished the degree of systole with arrest of the heart.

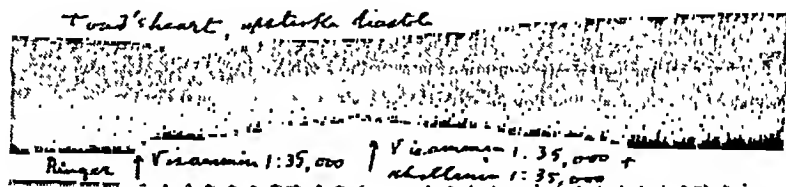


FIG. 4 (reduced).—Triple perfusion of a toad's heart, first with Ringer's solution then with visammin 1:35,000 and lastly with the same solution of visammin in which khellinin is dissolved in the same concentration. Depression of heart beat principally due to a less complete systole with visammin and recovery with stimulation under visammin and khellinin together are demonstrated. The amplitude of beat is greater under visammin and khellinin together than under Ringer's solution. Upstroke diastole.

The continued perfusion of the same solution of visammin to which khellinin was added to a concentration of 1:20,000 produced a fairly good recovery of the arrested heart with promotion of systole.

Similar triple heart perfusion experiments were carried out using chloral hydrate, quinine hydrochloride or alcohol in suitable concentrations against the same concentration of each one of these drugs in which khellinin was dissolved (1:25,000 to 1:50,000). depression of the heart beat was first established with subsequent stimulation in the presence of the glycoside.



FIG. 5 (reduced)—Triple perfusion of a toad's heart, first with Ringer's solution then with visammin 1:10,000 and lastly with the same solution of visammin in which khellinin 1:20,000 is dissolved. Under visammin alone, systole is greatly reduced with final arrest of the heart, while under visammin and khellinin together, recovery with promotion of systole is seen. Upstroke diastole.

(C) *Isolated rabbit's heart perfusion:* The heart was perfused by a modification of Gunn's method. Pressure of perfusion was kept at 100 to 120 mm. Hg. Outflow was determined every 2 minutes and 5 readings were registered for each change of liquid. In all perfusions partial experimental spasm was induced by the use of barium chloride and for this a concentration of 1:40,000 was maintained throughout—barium chloride Locke's solution was first perfused followed by the same solution of barium chloride containing either visammin (1:40,000) or khellinin (1:40,000) and lastly barium chloride Locke's solution. Only 6 experiments were carried out with the one concentration of visammin and similarly 6 experiments with khellinin giving respectively an average increase in coronary flow of about 200 and 300 per cent. Owing to the limited number of experiments carried out and, moreover, on one concentration only, no conclusion could be drawn as to their relative values in this respect.

Experiments on the intact animal and on the isolated toad's heart similar to those mentioned under (A) and (B) respectively but using various crude samples—obtained from the plant in various stages of impurity—of visammin and of khellinin gave variation and ambiguity of results. In many instances the cardiac depression of visammin or the cardiac stimulation of the glycoside was marked to a great extent. This, however, is very natural, *Ammi Visnaga* contains quite a number of active bodies. Products which differed from the pure principles by a few degrees in m.pt. gave quite appreciable differences in the response of the heart which is a fairly sensitive organ to these bodies.

Indeed, in addition to determination of m.pt., it would be wise to confirm the purity of material biologically by heart perfusion experiments.

SUMMARY

1. Pure khellinin, m.pt. 175° to 176°C., possesses a persistent, rather selective stimulant action on the heart producing a more

complete systole and a more complete diastole with a corresponding increase in cardiac output. It raises blood pressure. The glycoside is active and doses of 1 to 1.5 mg./kg. of dog by intravenous injection. It increases the coronary flow, and is non-cumulative.

2. Pure visammin, m.pt. 153° to 154°C., depresses the heart, producing principally a less complete systole with diminished cardiac output. The drug is active in doses of 2 to 4 mg./kg. of dog by intravenous injection. It increases the coronary flow.

3. The impurities present in crude samples of khellinin or of visamin influence to an appreciable degree the normal response of the heart to the pure products.

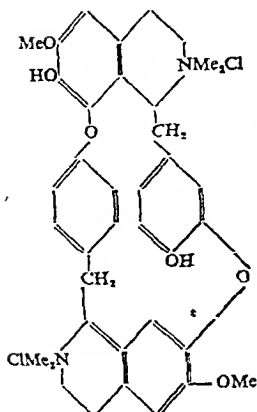
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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS



Curare Alkaloids, Constitution of *dextro*-Tubocurarine Chloride. H. King. (*J. chem. Soc.*, 1948, 267.) *dextro*Tubocurarine chloride, on O-ethylation, gave amorphous O-ethyltubocurarine chloride which when submitted to a two-stage Hofmann degradation gave the nitrogen-free O-ethylbebeerilene identical in properties with the substance obtained from bebeerine. The same distribution of methoxy- and phenolic groups is therefore present in *dextro*tubocurarine chloride and bebeerine; since their particular orientation is known in bebeerine, *dextro*tubocurarine chloride must have the structure shown.

R. E. S.

ANALYTICAL

Antihistaminic Drugs of the Thenyl Series, Identification of. T. J. Haley and G. L. Keenan. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 85.) The optical crystallographic properties and physical constants of antihistaminic drugs of the thenyl series are recorded. Those studied were thenylene or histadyl (N:N-dimethyl-N-(2-pyridyl)-N-(2-thenyl)-ethylenediamine) and its halogenated derivatives, chlorothen (N:N-dimethyl-N-(2-pyridyl)-N-(5-chloro-2-thenyl)-ethylene diamine) and bromothen (N:N-dimethyl-N-(2-pyridyl)-N-(5-bromo-2-thenyl)-ethylenediamine). A means for the identification and differentiation using 6 common alkaloidal colorimetric reagents is described. Tests were made by placing a drop of reagent on a microscope slide and adding about 1 mg. of the drug to it. Changes taking place were observed for about 30 minutes. The alkaloidal colorimetric reagents gave better results than three precipitation reagents also investigated but as the tests were almost identical for each of the thenyl compounds the optical crystallographic properties described offered the best means for their identification.

G. R. K.

Barbiturates; Xanthidrol as an Identification Reagent. R. S. McCutcheon and E. M. Plein. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 24.) Xanthidrol may be prepared by treating an alcoholic solution of xanthone with sodium amalgam or by reducing xanthone with zinc dust, Xanthone may be prepared by refluxing phenyl salicylate and distilling the product at high temperature. Xanthyl derivatives of 18 barbiturates were obtained by reaction of the barbiturates with xanthidrol in glacial acetic acid. Pure white crystals with characteristic m.pts. were obtained for most, clearly identifying these barbiturates. Two of the group of 20 studied were N-substituted barbiturates and did not react. The derivatives were dried to constant weight and analysed for nitrogen by the Kjeldahl-Gunning method. Deviation of the percentages found from those calculated did not exceed

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0.20 except for barbitone (calculated 5.15, found 4.86). M.pts. were determined by both the block and U.S.P. methods. The former checked consistently and were useful; rate of heating was 0.5°C. per minute. G.R.K.

Paper Chromatography, Streaming Potential in. L. Rutter. (*Nature* 1949, 163, 487.) The net resultant flow of solvent through the capillary channels of paper used in chromatography is in a direction away from the point of feed, resulting in the setting up of a streaming potential. Distilled water flowing through a washed and dried strip of No. 3 Whatman paper showed a potential gradient of approximately 10 mV. per cm. With a 1 per cent. thorium nitrate solution in place of water, the potential was reduced almost to zero; 1 per cent. sodium chloride solution showed a potential gradient of approximately 4 mV. per cm. The rate of flow of liquid and the nature of the electrodes used for measurement affect the values. Such potentials effect chromatographic development and it was found that development of 0.01 ml. of 0.1 per cent. aqueous solution of a mixed colour (Edicol Green 37113) with distilled water failed to separate the components, the band moving with the solvent front, whereas development with 1 per cent. sodium chloride solution achieved complete separation into blue and yellow bands. With either the strip or central feed technique, the flow of one liquid over another in partition chromatography may result in streaming potentials of varying sign, of possible significance in considering mechanisms of partition separations. R. E. S.

Paper partition Chromatography, Deposition and Simultaneous Concentration of Dilute Solutions in. K. F. Urbach. (*Science* 1949, 109, 259.) The concentrations of histamine encountered in ordinary paper partition chromatographic procedures, where 0.01 to 0.1 ml. volume of fluid is used, were too low relative to the sensitivity of the colour reaction used as indicator in the development of the chromatogram; a procedure was devised therefore so that the entire 3 to 5 ml. of butyl alcohol extract could be deposited without allowing excessive spreading of the solvent on the strips. Horizontal paper strips were fixed over a hot plate and maintained at 60° to 70°C. The extract was then dropped on to the strip from a capillary ended tube at a controlled rate of 1 ml. per hour, at which rate the solvent evaporates. The total spread of the spots is not more than 2 to 3 cm. Details of the apparatus and capillaries, together with diagrams are given. Other organic solvents, such as ether, acetone, alcohol, dioxane, etc., can be applied in a similar manner, although aqueous solutions spread excessively on the paper; solutions containing weak hydrochloric or sulphuric acid charred the paper even at moderate temperatures. R. E. S.

Stilbæstrol, Polarographic Studies of. L. E. Bingenheimer Jr. and J. E. Christian. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 117.) Electrolysis of solutions of stilbæstrol in concentrations of 10^{-4} to 3×10^{-4} M failed to give polarographic oxidation or reduction waves when the supporting electrolyte was N/10 potassium chloride in alcohol (30 per cent.) or in N/10 potassium hydroxide. Stilbæstrol suppressed the oxygen maximum in N/1000 potassium chloride solutions in alcohol (10 per cent.) or N/100 potassium hydroxide. The suppression was complete when the concentration of stilbæstrol was as little as 10^{-5} molar but negligible when the concentration was reduced to 10^{-6} molar. Repeated electrolysis decreased the size of the maxima and lowered the pH; buffering did not counteract this

effect but interfered, since the buffer exerted a suppressive action. Tablets of stilbœstrol were extracted by the U.S.P. method except that an extra step was necessary to remove gelatin which interfered with the results. The residue after removal of the ether was taken up in sodium hydroxide solution, potassium chloride was added and the solution diluted to correspond to 0.4×10^{-5} M stilbœstrol in N/100 potassium chloride and N/1000 potassium hydroxide. Standards corresponding to the U.S.P. limits of 90 and 110 per cent. of the labelled strength were similarly prepared. When the solutions were saturated with oxygen and electrolysed, the size of the maximum in the unknown solution was between those in the standard solutions. The main advantage of this method over the U.S.P. method is the saving of about 1 hour.

G. R. K.

Tragacanth Flake, Evaluation of. Report No. 2 of the Tragacanth Subcommittee of the Analytical Methods Committee of the Society of Public Analysts. (*Analyst*, 1949, 74, 2.) The following recommendations are made: *Viscosity*—The flake is ground rapidly until all passes a No. 30 mesh sieve and a quantity of the powdered gum equivalent to the required weight of dry gum is wetted with 5 ml. of 95 per cent. alcohol. Cold distilled water is then added quickly, the mixture is shaken, allowed to stand for 1 hour and is then heated in a boiling water bath, the determination being completed as described in Report No. 1 (*Analyst*, 1948, 73, 368; *J. Pharm. Pharmacol.*, 1949, 1, 44.). *Suspending Power*—Owing to the variations in suspending power in gums of the same viscosity it is recommended that the purchaser should carry out a form of test using the concentration of tragacanth normally employed in his process and using all the materials that he desires to suspend. *Ash*—Direct ashing failed to give concordant results and, after preliminary treatment the sulphated ash (at about 850°C .) was chosen. *Volatile acidity*—The method used was described in "Methods of Analysis of the Association of Official Agricultural Chemists," 6th Edition, 1945, p. 709. The detailed procedure necessary to obtain concordant results is given in each case.

R. E. S.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Digitalis Glycosides, Chemistry and Pharmacology of. E. W. McChesney, F. C. Nachod, M. E. Auerbach and F. O. Laquer. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 364.) Analytically pure samples of gitoxin, digitoxin and their aglucones have been prepared. Experiments on rats showed gitoxin to be only slightly less toxic than digitoxin. It is known that gitoxigenin gives a red colour with ferric chloride in the presence of strong sulphuric acid, the intensity of the colour increasing rapidly up to 5 minutes and then fading. Without the ferric chloride the red colour develops slowly, reaching a maximum in about 24 hours and remaining unchanged for several days. Digitoxigenin treated similarly gives a pale lemon-yellow colour which gradually deepens. The glycosides give similar colour reactions but the aglucones are much better adapted to colorimetric work, since with the glycosides there is admixed a brown colour resulting from the reaction of sulphuric acid on the digitoxose. The absorption spectra of the aglucones in sulphuric acid were studied and their different behaviour was found to provide the basis for an analytical method which gives the proportion of digitoxin and gitoxin plus gitalin in a mixture.

S. L. W.

ABSTRACTS

ESSENTIAL OILS

Ascaridol, Assay of; Iodination of Terpenes. A. Halpern. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 465.) The assay of ascaridol in oil of chenopodium depends on the liberation of iodine from potassium iodide. The author shows that the major part of the iodine is released within the first minute; this is followed by a slower steady release of iodine. In order to elucidate whether the olefinic linkage was responsible for this property an attempt was made to iodinate several related unsaturated hydrocarbons under conditions similar to those of the assay process. No iodination of the olefinic linkage occurred, so that the atypical behaviour of ascaridol toward the iodide reagent cannot be explained on this basis.

J. W. F.

GUMS AND RESINS

***Sterculia setigera*, Composition of the Gum of.** E. L. Hirst, L. Hough and J. K. N. Jones. (*Nature*, 1949, 163, 177.) The analysis of reducing sugars by means of paper partition chromatography yields inconclusive results for mixtures of sugars with very similar R_g values. The use of a column of powdered cellulose was found to afford a method for the separation of sufficiently large amounts of the individual sugars to allow identification by the normal determination of physical constants; results are given from a study of the gum of *Sterculia setigera*. Partial hydrolysis of the gum gave a mixture of sugars and a degraded material containing the uronic acids. The uronic acid portion had properties corresponding to a trisaccharide containing two D-galacturonic acid residues and a sugar residue, probably mainly L-rhamnose. The chief components of the non-acidic portion were D-galactose, L-rhamnose and a ketose, which, from its properties, could be fructose, tagatose or sorbose. A column of powdered cellulose was used to separate the sugar mixture with a solution of *n*-butyl alcohol saturated with water as the mobile phase. Two fractions crystallised spontaneously yielding D-galactose and L-rhamnose hydrate; D-tagatose was obtained from an intermediate fraction after oxidation of the accompanying aldose to the aldonic acid, which was removed as the barium salt. The D-tagatose thus isolated was identical with the sugar prepared synthetically. In all, five fractions were obtained containing respectively: a ketose, probably a methyl pentose: L-rhamnose; an aldose and D-tagatose; an aldose, tagatose and D-galactose: and D-galactose; D-tagatose has not hitherto been reported as a constituent of any natural product.

R. E. S.

ORGANIC CHEMISTRY

***p*-Aminobenzoic Acid and its Sodium Salt.** C. J. Kern, T. Antoshkiw and M. R. Maicse. (*Anal. chem.*, 1948, 20, 919.) Sodium *p*-aminobenzoate was purified by charcoal treatment and three recrystallisations from aqueous solution. Purified *p*-aminobenzoic acid, prepared from the sodium salt by precipitation with hydrochloric acid, washing and drying at 100°C., melted at 187° to 187.5°C. A curve is given of the pH changes during titration of the acid with standard alkali and the equivalence point of 7.85, $pK^a=4.65$ is derived. For acidimetric determination an indicator with a colour change between 7.0 and 8.7 is therefore suitable. A characteristic absorption spectrum was obtained in isopropyl

alcohol, wave length maximum = 288m μ . $E_{1\text{ cm.}}^{1\text{ per cent.}} = 1370$. In water both *p*-aminobenzoic acid and its sodium salt show about the same characteristic wave length, maximum = 266m μ , and $E_{1\text{ cm.}}^{1\text{ per cent.}} = 1070$, Beer's law being obeyed in both isopropyl and aqueous solutions. A table is given of comparison of results obtained by the spectrophotometer, titration, and diazo methods, all three being suggested for complete characterisation of pure *p*-aminobenzoic acid.

R. E. S.

2-Amino-3-hydroxybenzoic Acid, Synthesis of. J. F. Nye and H. K. Mitchell. (*J. Amer. chem. Soc.*, 1948, 70, 1847.) This substance was synthesised by two routes. In the first, 2-nitro-3-methoxybenzoic acid was reduced by catalytic hydrogenation to 2-amino-3-methoxybenzoic acid, followed by demethylation with hydriodic acid. The second method involved oxidation of 8-methoxyquinoline to give 2-(*N*-methyl-*N*-formyl)-amino-3-methoxybenzoic acid; appropriate treatment with hydriodic acid gave 2-amino-3-hydroxybenzoic acid m.pt. 254 to 255° C. (corr.). The compound of Keller (*Arch. Pharm.*, 1908, 246, 1) reported as 2-amino-3-hydroxybenzoic acid was in reality the 3-methoxy derivative. Graphs of the ultra-violet absorption spectra of the 3-hydroxy and 3-methoxy derivatives are given.

R. E. S.

Mercurial Derivatives of Sulphanilamide. G. Rodighiero. (*Ann. Chim. appl. Roma.*, 1949, 39, 27, 34.) In organic mercurial compounds the bond C-Hg usually shows greater resistance to reagents than the bond N-Hg and this influences the toxicity, antisyphilitic action and the uses in therapy and hygiene of these compounds. Mercurial derivatives of sulphanilamide are particularly interesting since they give the possibility of the mercury being linked to a carbon atom of the aromatic nucleus or to amino nitrogen or amido nitrogen. One molecule of sulphanilamide with 1 molecule of sodium hydroxide and 1 molecule of mercuric acetate gives (A) $\text{H}_2\text{N.C}_6\text{H}_4\text{SO}_2\text{NH.HgOH}$. This is a white powder, decomposing on heating, insoluble in water, organic solvents and alkalis, but dissolving in hydrochloric acid to give the chloride (B) $\text{HCl.H}_2\text{N.C}_6\text{H}_4\text{SO}_2\text{NH.HgCl}$. This can be crystallised, has m.pt. 155° to 157°C. and is soluble in alcohol. These compounds have a notable antibacterial activity. The substance B dissolved in water and 0.5 to 1 molecule of sodium nitrite added at 0°C. gives (C) $\text{HOHg.HNO}_2\text{SC}_6\text{H}_4\text{NH}=\text{NC}_6\text{H}_4\text{SO}_2\text{NH.HgOH}$. This is a yellow powder insoluble in water, organic solvents and alkalis, but dissolves in hydrochloric acid, the solution liberating nitrogen on warming. The substance A, dissolved in 4 or more molecules of hydrochloric acid gives on the addition of 1 molecule of sodium nitrate at 0°C. (D) $\text{H}_2\text{N.SO}_2\text{C}_6\text{H}_4\text{N}=\text{NCl.HgCl}_2$. This is a white powder which can be crystallised from water; soluble in acetone and alcohol, insoluble in ether, benzene, and chloroform. It deflagrates on heating. One molecule of the substance D dissolved in acetone and shaken with 2 molecules of powdered copper gives (E) $\text{H}_2\text{N.O}_2\text{S.C}_6\text{H}_4\text{.HgCl}$. Crystallised from acetone, this occurs in white needles, melting with decomposition at 315° C. It is insoluble in water and dilute acids, but soluble in sodium hydroxide, being reprecipitated unaltered on acidification. It is insoluble in ether and light petroleum, slightly soluble in hot alcohol, readily soluble in pyridine. If the substance E is dissolved in pyridine and poured into water a white amorphous unstable compound is precipitated, which when kept in a vacuum desiccator over sulphuric acid finally loses its

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pyridine leaving (F) $C_6H_4.SO_2NH$. This is a white, amorphous powder insoluble in water, acids, bases and organic solvents, except pyridine, slightly soluble in acetic acid. It does not melt on heating and is unchanged at $320^\circ C$.
H. D.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Aminobenzoic Acids, Production of, by Sulphonamide-resistant Bacteria. R. Lemburg, J. P. Callaghan, D. E. Tandy and N. E. Goldsworthy. (*Austral. J. exp. Biol.*, 1948, 26, 9.) It is widely held that drug-resistance can be explained by an increased rate of production, or an increased total production, of *p*-aminobenzoic acid by a resistant strain of bacteria as contrasted with the susceptible parent strain. The main difficulty in demonstrating this has been the lack of specific methods for the isolation of *p*-aminobenzoic acid from the bacterial cultures. Attempts have therefore been made to develop a method of extraction and separation of aromatic amino-acids which would be sufficiently sensitive to be used with the low concentrations of these substances found in bacterial cultures. The method adopted by the authors consisted in the transformation of the aromatic amino-acids into azo-dyes by diazotisation and coupling with dimethyl- α -naphthylamine, in the separation of the acidic from the non-acidic dyes, and in separation of the former by chromatography on alumina. A strain of *Bacterium coli*, growing in synthetic Kisch's medium, produced diazotisable amino-acids in the supernatant; these were identified for the most part as anthranilic acid and *p*-aminobenzoic acid. After this strain had become adapted to sulphathiazole it did not produce more *p*-aminobenzoic acid than the parent susceptible strain, and the amount of *p*-aminobenzoic acid was insufficient to account for the resistance to sulphathiazole. The same held for the sum of *o*- and *p*-aminobenzoic acid produced. The production of anthranilic acid was variable and decreased with the number of sub-cultures. The authors therefore concluded that *p*-aminobenzoic acid production does not explain acquired drug-resistance so far as *Bacterium coli* is concerned. It may be true that the method of drug adaptation is different in different organisms.
S. L. W.

Antibiotic Activity, as Shown by a Highly Amylolytic Strain of *Bacillus subtilis* B. S. Lulla. (*Nature*, 1949, 163, 489.) *Bacillus subtilis* when grown on wheat bran medium, showed a pronounced antibiotic activity, the maximum being found in the aqueous extract from a 24-hour old culture; during this period the amylase formation was found to be low, but steadily increased with further incubation. Results are given of a study of the relationship between the antibiotic production and amylase formation by *B. subtilis* (N.C.T.C.:2027 N) when grown on wheat bran. The antibiotic activity, at a maximum on the first day of growth, gradually disappears with further incubation, while amylase production, although negligible on the first day, steadily increases as the incubation period proceeds, and reaches its peak value on the fourth day. There is therefore a relationship between amylase formation and the production of antibiotic substance.
R. E. S.

Insulin, Regeneration from Insulin Fibrils by the Action of Alkali. D. F. Waugh. (*J. Amer. chem. Soc.*, 1948, 70, 1850.) Reversion by alkali treatment of insulin fibrils produces a crystalline product (termed r-insulin) similar to native insulin. A detailed method is given for the complete conversion of insulin into freely suspended insulin fibrils by heating crystalline zinc insulin at 100°C. with 0.05N hydrochloric acid in sealed glass ampoules. Limiting conditions for regeneration procedure were determined by studying the effect of alkali on native insulin. Using 0.5 ml. of 2 per cent. insulin (10 mg.) and 5.0 ml. of sodium hydroxide, experiments indicated that 0.03N alkali, 0°C. and a 45-minute treatment time gave the best results. Reversion of the fibrils was greatly accelerated by increasing the number of available fibril ends (by a freezing and thawing cycle which breaks up the longer fibrils into short segments), suggesting that disaggregation occurred mainly at these positions. The presence of sodium chloride in N/1 concentration in the alkali caused a 90 per cent. inhibition of reversion; the repulsive forces between similarly charged groups may thus play a part in the mechanism of disaggregation. The crystalline product from reverted fibrils was not found to be significantly different from native insulin in crystallisation properties, in biological activity (20 I.U. per mg.), in ultracentrifuge pattern (sedimentation constant 3.3 to 3.6), and in fibril formation (at 20° and 100°C.). The irreversible loss of one or more of the characteristic properties following demonstrable changes in internal structure was not found with r-insulin; tests for changes in labile groups, such as amino and disulphide, have been negative. The retention by r-insulin of characteristic insulin properties known to be sensitive to structural changes, the absence of changes in labile groups, and the fact that fibril elongation may take place at low temperatures in the pH region of maximum stability, are interpreted as showing that only small structural changes take place during fibril formation and that the process is one in which globular or corpuscular units are linked endwise.

R. E. S.

Neomycin, a New Antibiotic. S. A. Waksman and H. A. Lechevalier. (*Science*, 1949, 109, 305.) The organism producing neomycin was isolated from the soil, and is related to *Streptomyces fradiae*. When the newly isolated culture was grown in various media containing a source of nitrogen, a carbohydrate, and salt, it was found to produce neomycin under both stationary and submerged conditions of culture. The antibiotic can easily be removed from the culture medium and concentrated by the methods of adsorption and elution applicable to streptomycin. Neomycin is a basic compound, most active in an alkaline medium. It is soluble in water and insoluble in organic solvents. It is thermostable. It is active against numerous Gram-positive and Gram-negative bacteria, especially mycobacteria, but not against fungi. The antibiotic spectrum of crude neomycin is quite distinct from that of streptomycin or streptothricin. Neomycin preparations were found to possess the following desirable properties: (1) similar activity against both streptomycin-sensitive and streptomycin-resistant bacteria; (2) considerable activity against various forms of -resistant mycobacteria; (3) limited or no toxicity to animals; (4) activity against various bacteria *in vivo*, including Gram-positive and Gram-negative organisms and against both streptomycin-sensitive and streptomycin-resistant organisms; (5) lack of resistance against neomycin among the organisms sensitive to it, or only limited development of such resistance. Neomycin has not

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yet been obtained in crystalline form, but preliminary results point to its being distinctly different chemically from streptothricin and from streptomycin.

S. L. W.

Penicillin, Diffusion of. L. Mosonyi, R. Held and Ch. Kocsán, (*Acta med. scand.*, 1942, 132, 487.) The diffusion properties of penicillin are different from those of other crystalline substances. It is evenly absorbed by colloidal substances, such as agar, without losing its efficiency and its diffusion is greatly influenced by this adsorption. A thrombin-fibrinogen membrane was shown to exert an adsorptive action on penicillin similar to that of agar, a fibrin layer of 3 mm. preventing the action of 0.5 to 1 unit of penicillin when interposed between the penicillin and an infected agar plate, though a fibrin layer of this thickness is not able to inhibit the action of penicillin when used in larger quantities (5 to 10 I.U.). As the concentration of penicillin in the blood, even when given intravenously, reaches only 0.3 to 0.4 units, and this for a very short time only, this explains why bacteria at the base of vegetations such as occur on the endocardium in endocarditis, and which often exceed a thickness of 4 or 5 mm., are found to retain their full virulence in spite of administration of penicillin. This adsorptive action of fibrin can be lessened, and the penicillin rendered more diffusible, by the addition to the penicillin solution of 20 per cent. of sodium dehydrocholate.

S. L. W.

Penicillin. Enhancement of Therapeutic Activity. G. A. Hobby, T. F. Lenert, W. Reed and D. Renne. (*J. Bact.*, 1949, 57, 247.) As a result of further work to discover the reason for the enhanced activity of impure penicillin as compared with crystalline penicillin G, evidence has been found suggesting that certain degradation products of penicillins G and dihydro-F respectively enhance the activity of highly purified samples of the two penicillins. The products possibly responsible are *p*-hydroxyphenylacetic acid, caprylic acid, penillic and penicilloic acids.

H. T. B.

Progesterone, Stability of. R. B. Woolf and W. M. Allen. (*Proc. Soc. exp. Biol., N.Y.*, 1948, 67, 79.) Samples of α and β -progesterone isolated from pigs' ovaries in 1936 were found on assay to be as active, after storage for 10 years, as the first preparations obtained by Wintersteiner and Allen in 1934. The preparations had been stored in small, unsealed glass-stoppered vials at room temperature. Both α and β -forms were assayed according to a slight modification of the original Corner-Allen method and the results were compared with the data obtained in 1934 by the original method. The comparison showed that the quantities necessary to produce full proliferation, and the quantities which produced little or no proliferation, were virtually the same, establishing beyond doubt that there had been no great change in activity in the interim. The dosage response curves from the data of 1934 were found not to differ significantly from those obtained from assay in 1946.

S. L. W.

Steroids, Deuterium-labelled. Infra-red Spectrometry in Metabolic Studies. K. Dobriner, T. H. Kritchevsky, D. K. Fukushima, S. Lieberman, T. F. Gallagher, J. D. Hardy, R. N. Jones and G. Cilento. (*Science*, 1949, 109, 260.) Infra-red absorption spectra of steroids, with one or more of the hydrogen atoms replaced by deuterium, are of value in the detection, analysis and identification of these compounds. The spectrum of pregnanol-3-(α)-one-20 is compared with the spectrum of the same compound where a hydrogen atom at C-11 and at C-12 has been

replaced by deuterium. Two absorption bands appear at 2,165 and 2,125 cm^{-1} in the deuterium-containing compound and in the neighbourhood of 1,200 cm^{-1} pronounced differences are apparent in the two spectra. The C-D absorption bands in the neighbourhood of 2,150 cm^{-1} are useful for the identification of a deuterium-containing molecule as this region, in the concentrations used, is transparent in the absence of deuterium. The method was very sensitive and the presence of deuterium could be established in as little as 25 μg . of pregnan-11:12- d_2 -ol-3-(α)-one-20 containing 5 atoms per cent. excess of the isotope. In a metabolic experiment allopregnan-5,6- d_2 -ol-3-(β)-one-20-acetate was injected into a normal woman and the urine and faeces were collected over 20 days; a fractionation procedure for treatment of urine and faeces followed by spectrum examination showed that the isotope was present in both the crude α - and β -hydroxy-ketonic fractions as well as in the α - and β -hydroxy non-ketonic fractions from both urine and faeces. Graphs are given of the infra-red spectra of normal and deuterium steroids in carbon disulphide solution for pregnanolone and pregnan-11:12- d_2 -ol-3-(α)-one-20.

R. E. S.

Tricothecin, Isolation and Chemical Properties of. G. G. Freeman and R. I. Morrison. (*Biochem. J.*, 1949, 44, 1.) The isolation of tricothecin from the culture filtrate of *Tricothecium roseum* is described. The nitrate was extracted with chloroform and the residue after evaporation of the chloroform was dissolved in ether and fractionated on a column of activated alumina using ether to develop the column. After fractional precipitation of inactive material from light petroleum and chloroform, the residue obtained was dissolved in carbon tetrachloride and again fractionated on an alumina column until pure tricothecin, m.pt. 118°C., was obtained. The substance dissolved in chloroform, ethyl alcohol, acetone, and benzene, and was slightly soluble in water (400 mg./l. at 25°C.); it had optical activity $[\alpha]^{18^\circ\text{C}} + 44^\circ$ (c.1, in chloroform); analytical data were consistent with the molecular formula $\text{C}_{15}\text{H}_{18}\text{O}_4$ or $\text{C}_{15}\text{H}_{20}\text{O}_4$. The molecule contained one ketone group, one ethylenic group and three methyl groups attached to carbon; free carboxyl, hydroxyl, alkoxyl and aldehyde groups were absent. On hydrolysis with alcoholic potassium hydroxide, tricothecin combined with one equivalent of alkali. The ultra-violet absorption spectrum of tricothecin contained two main bands which, together with the shift of the bands occurring with a change in polarity of the solvent, indicated that the molecule contained conjugated ethylenic and carbonyl groups. Tricothecin was found to be relatively stable in acid solution and at pH 10, but at pH 12 hydrolysis took place with liberation of a carbonyl group and with virtually complete loss of antifungal activity in 6 hr. at 20°C. Acidification of the inactivated alkaline solution led to the formation of an inactive neutral ketone.

R. E. S.

BIOCHEMICAL ANALYSIS

Alcohol in Blood and other Biological Fluids, Colorimetric Determination of Microquantities of. R. J. Henry, Carol F. Kirkwood, S. Berkman, R. D. Housewright and J. Henry. (*J. Lab. clin. Med.*, 1948, 33, 241.) The method depends upon the oxidation of the alcohol to acetaldehyde and determination of the latter colorimetrically with *p*-hydroxydiphenyl. Up to 20 ml. of sample is diluted with 20 ml. of water and 0.1 ml. of 10 per cent. sodium hydroxide solution and distilled. The

ABSTRACTS

distillate, which should contain all the alcohol, is added to a solution of potassium dichromate and sulphuric acid and again distilled. The distillate is collected in a graduated tube in an ice-bath, the quantity collected depending on the amount of alcohol in the original sample. The loss of alcohol by complete oxidation to acetic acid is controlled by strict adherence to the routine described. The acetaldehyde is estimated by adding to 1 ml. of the distillate cooled in ice, 1 drop of 5 per cent. copper sulphate solution, 6 ml. of arsenic and nitrogen-free sulphuric acid, and, with constant shaking, 0.1 ml. of a 1.5 per cent. solution of *p*-hydroxydiphenyl in 0.5 per cent. sodium hydroxide solution. The mixture is warmed at 30° C. for 30 minutes, placed in a boiling water-bath for 90 seconds to dissolve excess of reagent, and cooled in ice to room temperature. The deep violet colour, which is stable for several hours, is read in a photoelectric colorimeter against a blank prepared by using 1 ml. of water instead of the distillate. Substances which interfere with the results include oxalacetic acid, α -glycerophosphates, glyceraldehyde and other alcohols. Among the non-interfering substances listed are methyl alcohol, glucose, acetone, pyruvic acid, urea and various amino-acids. The method is suitable for the determination of blood in amounts obtainable from finger puncture. The blood should be diluted 1 in 20, and the proteins and erythrocytes precipitated with tungstic acid. The method gives results within ± 6 per cent. on a single blood determination.

G. R. K.

Alcohol, Ether and Volatile Reducing Substances in Blood and Gases, Determination of. A. Hemingway, L. A. Bernat and J. Maschmeyer. (*J. Lab. clin. Med.*, 1948, 33, 126.) Various methods used for the determination of alcohol and ether in blood and air are reviewed and their disadvantages are examined. In the procedure adopted the reducing substance is absorbed in a known excess of standard potassium dichromate solution in the presence of sulphuric acid and the excess of chromic acid is determined by titration with ferrous sulphate solution using the redox indicator barium diphenylamine sulphonate. In the presence of phosphoric acid the indicator gave a sharp end-point from violet-blue to colourless, which is the main advantage of the method. Details are given for the determination of reducing substances in small quantities of blood using the Widmark flask method, and also for the determination in gases. The effect of variation in distillation time is studied and results are given of tests carried out on the reducing power of sulphuric acid, on the oxidation of acetic acid, and on the recovery of ether from prepared solutions of ether in blood.

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***p*-Aminosalicylic Acid in Blood and Urine, Estimation of.** H. G. Dickenson and W. Kelly. (*Lancet*, 1949, 256, 349.) To a solution of 1 per cent. sulphanilic acid in 10 per cent. hydrochloric acid, cooled in ice, add 10 per cent. sodium nitrate solution until the reaction is just positive to starch iodide; then add the sulphanilic-acid solution until the starch iodide test is negative. This solution is kept cold and made up freshly for each test. Blood serum (2 ml.) containing *p*-aminosalicylic acid equivalent to 10 to 20 mg./100 ml. diluted with water (2 ml.) is deproteinized with 10 per cent. trichloroacetic acid (2 ml.), and filtered. The filtrate (2 ml.) is made strongly alkaline by adding 30 per cent. sodium hydroxide solution (0.25 ml.) and the diazo solution (0.25 ml.) added. The stable cherry colour produced is compared with that obtained from standard aqueous solutions of *p*-aminosalicylic acid equivalent to 10 to 20

mg./100 ml. Sulphonamides and *p*-aminobenzoic acid give no coloration, and normal blood only a negligible coloration. Salicylic acid gives a coloration only about 5 per cent. of that obtained for *p*-aminosalicylic acid. The recovery of added *p*-aminosalicylic acid from blood and blood serum is from 90 to 100 per cent. For the estimation in urine, 5 to 10 drops of a 10 per cent. calcium chloride solution is added to an aliquot (10 ml.) of the urine sample, the pH brought to 8 to 9 with 3N ammonia, the solution filtered, and the filtrate and washings adjusted to pH 2 with 10 per cent. hydrochloric acid. The intensity of colour on adding 2 drops of 10 per cent. ferric chloride solution is observed on a sample, and the urine diluted to give a colour approximately equivalent to that obtained from a standard solution of *p*-aminosalicylic acid. A colorimetric estimation can be made with an accuracy of ± 5 per cent. Salicylic acid will, of course, interfere with this estimation.

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Anti-Pernicious Anæmia Factor. Estimation of. W. F. J. Cuthbertson. (*Biochem. J.*, 1949, 44, v.) The microbiological assay of Shorb for the growth factor present in highly refined liver extracts was found to be unsatisfactory. The organism used, *Lactobacillus lactis* Dorner ATCC 8000, required, in addition to the medium of Shorb, tomato juice and "Tween 80" as well as the anti-pernicious anæmia factor. Thymidine allowed the growth of the organism on vitamin B₁₂-deficient media and it was not possible to obtain a response to the anti-pernicious anæmia factor using the technique of Shorb. The cup-plate assay was adaptable to the determination of the anti-pernicious anæmia fraction and to the detection of other members of the B₁₂ group of microbiological growth factors present in purified liver extracts. The medium used was that found suitable in the ordinary microbiological assay with the addition of 2 per cent. of agar. For an assay the sterile medium is melted, held at 45°C., and inoculated with a culture of *Lb. lactis* Dorner ATCC 8000; 12.5 ml. samples are then poured into Petri dishes. Holes are cut in the covered agar plates with a 10 mm. cork borer. Three drops of test or standard solution are placed in each of the appropriate holes and the plates are incubated overnight. After 16 to 24 hr. the colonies developing around holes form sharply defined zones of exhibition: zone diameters are proportional to the logarithms of the anti-pernicious anæmia fraction concentrations over the range 0.02 to 0.5 µg./ml. Both factors contribute to microbiological activity and unless the ratio of clinical to microbiological activity is the same for both of these substances this test alone will not exactly predict the clinical potency of liver extracts. The method is rapid and simple, but it is relatively insensitive and somewhat susceptible to interference by other members of the B₁₂ group, preservatives and antibiotics.

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Penicillin. Paper Strip Chromatography. R. G. Klueener. (*J. Bact.*, 1949, 57, 101) A modified paper strip technique which can be completed in 24 hours is described for determining separately penicillins X, G, F and K. The method is based on the differences in the distribution coefficients of the varieties of penicillin between ether and a phosphate buffer. The ether used for development must be anhydrous and of reagent grade. In analysing 35 known mixtures in buffer solution and 24 known mixtures in broth, the difference between percentages added and those found did not exceed 13 per cent. and averaged ± 5 per cent.

H. T. B.

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H. T. B.

Penicillin G, Determination of. G. B. Levy, D. Shaw, E. S. Parkinson and D. Fergus. (*Anal. Chem.*, 1948, 20, 1159.) The light absorption in the ultra-violet region due to the benzyl group in penicillin G, is used to determine this component in mixtures of penicillins. The total light absorption of a penicillin mixture is due to a "background" non-selective absorption upon which is superimposed the benzene band spectrum which can be evaluated by several methods; graphs are given illustrating this procedure. For routine analysis of penicillin G preparations, a simplified technique was used based on the fact that commercial penicillin G preparations usually do not contain penicillin X or inactivated penicillin G, but, besides benzylpenicillin, only small amounts of penicillins F, K, and dihydro F, and some inert pigment. Under these conditions an average value for the angular displacement of the spectrum due to impurities, i.e. the slope of the "background" absorption—together with the measurement of the height of a characteristic band (maximum and minimum) affords a rapid method for determining the benzyl content of penicillin G preparations. Comparisons of assays by this method with the gravimetric procedure, based on the precipitation of penicillin G by N-ethyl piperidine, showed that the average deviation for the gravimetric method is 1.43 per cent., and for the spectrophotometric method 1.40 per cent., while the maximum deviation is 4.5 and 5.2 per cent. respectively. The method evolved is particularly suited to routine use but extraneous benzyl groups will interfere. A photoelectric spectrophotometer is necessary to produce the required accuracy of extinction readings.

R. E. S.

Sodium in Biological Fluids, Microcolorimetric Determination of. A. A. Albanese and M. Lein. (*J. Lab. clin. Med.*, 1948, 33, 246.) The reagent is a solution of uranyl zinc acetate prepared by adding a boiling solution of 10 g. of uranyl acetate in 50 ml. of water and 2 ml. of glacial acetic acid to a boiling solution of 30 g. of zinc acetate in 50 ml. of water containing 1 ml. of glacial acetic acid, allowing to stand overnight, filtering, diluting with an equal volume of alcohol (95 per cent.), cooling at 4°C. for 48 hours and again filtering. A mixture of 0.2 ml. of urine or spinal fluid and 1 ml. of reagent is cooled at 4°C. for one hour, and centrifuged. The supernatant liquid is discarded, the residue drained and washed with 2 ml. of alcohol (95 per cent.), again centrifuged and drained, and dissolved in 5 ml. of water. Any turbidity due to an excess of phosphate is removed by centrifuging and the intensity of the yellow colour of the solution measured in a photoelectric colorimeter. A parallel determination is done for 0.2 ml. of a standard solution of sodium chloride in water containing 2 mg. of sodium per ml. The content of sodium per ml. is calculated from: reading of unknown/reading of standard \times 0.4 mg. of Na \times 5. For sera, plasma and whole blood, 0.2 ml. is treated with 0.6 ml. of a 20 per cent. solution of trichloroacetic acid, centrifuged, and 0.4 ml. of the supernatant liquid treated as described. The experimental error is about ± 5 per cent.

G. R. K.

Steroids in Urine, Determination of. S. L. Tompsett. (*Analyst*, 1949, 74, 6.) A review is made of the methods available for the isolation and identification of the steroids found in human urine. The origin of the principal steroid hormones, male and female, is given, together with a detailed table of the principal natural steroids found in man. Steroids in urine are separated from other materials by three procedures: (1) extraction of the steroid conjugates with butyl alcohol followed by acid hydrolysis, (2) a short acid

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hydrolysis followed by extraction of the free steroids with an organic solvent, (3) simultaneous hydrolysis by acid and extraction with a comparatively high-boiling solvent. Methods for the determination of total 17-ketosteroids, of non-alcoholic 17-ketosteroids, and of α - and non-alcoholic 17-ketosteroid content after precipitation of the β -alcohols with digitonin, are given. The 3:20-ketosteroid fraction is discussed and a method is given for the determination and separation of ketonic and non-ketonic steroids using Girard reagent T. Results are quoted for urine samples from a wide variety of clinical cases. For a complete picture of steroid hormone metabolism the following determinations are necessary: oestrogens; 17-ketosteroids; total ketones indicative of the presence of the 20-ketosteroids; the non-ketones; pregnanediol-3(α):20(α); the corticosteroids.

R. E. S.

CHEMOTHERAPY

Diamidines as Antibacterial Compounds. R. Wien, J. Harrison and W. A. Freeman (*Brit. J. Pharmacol.*, 1948, 3, 211.) In the diphenoxyalkanes there was a graded increase in bacteriostatic activity, which was maintained in the presence of blood, against staphylococci, rising to a maximum from the propane to the hexane and nonane derivatives. This increase was accompanied by an increase of intravenous toxicity but by only a relatively small increase in local toxicity to phagocytes. Gram-positive bacteria were more susceptible than Gram-negative bacteria. The introduction of halogen into one or both benzene nuclei in the diphenoxyalkanes increased bacteriostatic activity, with little alteration in local toxicity to phagocytes. The mono-halogen derivatives were more active than the di-halogen against staphylococci, whereas the di-halogen derivatives were more active against Gram-negative bacteria. Dibromopropamidine and iodoexamidine were amongst the most active of the compounds examined for their possible use in surface infections. They both showed bacteriostatic and bactericidal activity, these effects being decreased in an acid and increased in an alkaline medium. Drug-resistant strains of bacteria could easily be induced by repeated sub-cultivation *in vitro*. Cross-resistance experiments showed that: (1) staphylococci resistant to penicillin or to 5-amino-acridine were susceptible to diamidines, (2) staphylococci and streptococci resistant to one diamidine were resistant also to other diamidines, (3) staphylococci resistant to diamidines were not resistant to penicillin or 5-amino-acridine. Little therapeutic activity can be demonstrated when the compounds are given by injection.

S. L. W.

Miracil D. D. M. Blai, and F. G. Loveridge. (*Lancet*, 1949, 256, 344.) Miracil D was given by mouth twice a day for 3 to 6 days to African school-children. Of 82 children who received a total dosage of at least 60 mg./kg. of body weight 74 ceased to pass living eggs or active miracidia, and none of the cured cases had relapses up to 12 weeks after treatment. There seems to be no advantage in giving more than 15 mg./kg. daily. This dosage caused no symptoms in about half the children. The others complained of abdominal pain, loss of appetite, nausea, and/or headache and dizziness, but none were ill enough to seek medical aid. A few children infected with *S. mansoni* were also treated with the same dosage but the results were unsatisfactory.

S. L. W.

PHARMACY

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Cetyltrimethylammonium Bromide, Efficacy of in Ointment Bases. L. P. Prusak and A. M. Mattocks. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 67.) Six ointments comprising 1 per cent. of cetyltrimethylammonium bromide in hydrophilic ointment U.S.P., hydrophilic petrolatum U.S.P., simple ointment U.S.P., tragacanth glycerite N.F., pectin paste N.F., and a carbowax ointment base prepared by mixing carbowax 1500 with 10 per cent. of water, were added in amounts of 0.5 g. to 5 ml. quantities of nutrient broth inoculated with *Staphylococcus aureus*, and the mixtures were incubated at 37°C. for 3- and 24-hour periods. One loopful (0.05 ml.) of each mixture was sub-cultured in a medium containing lecithin, which inhibits the action of the bactericide, incubated for 48 hours and the tubes were read for turbidity. Control tests were carried out to determine the extent to which the ointment bases interfered with the action of the bactericide. Pectin paste and carbowax ointment base proved to be suitable ointment bases. The ointments prepared with hydrophilic ointment and hydrophilic petrolatum had no bactericidal activity, while those prepared with simple ointment and tragacanth glycerite released such small amounts of bactericide as to be practically ineffective.

G. R. K.

Saccharated Iron Oxide for Intravenous Administration. J. H. Nissim and J. M. Robson. (*Lancet*, 1949, 256, 686.) Samples of saccharated iron oxide differ widely in toxicity owing to differing methods of preparation. The following method permits of better control and provides material of lower toxicity. Dissolve 25 g. of anhydrous sodium carbonate in 1 l. of distilled water and add 50 g. of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$). Carbon dioxide is evolved and the ferric hydroxide formed dissolves in the excess of ferric chloride to give a dark purple solution. Add sodium carbonate solution gradually to reprecipitate all the iron as ferric hydroxide, and adjust to pH 7. Wash away the sodium chloride formed by repeated additions of distilled water in quantities of 1000 to 2000 ml., allowing the ferric hydroxide to settle and decanting, continuing until the supernatant fluid acquires a brownish tinge. Add 166 g. of sucrose, followed by 30 ml. of 15 per cent. sodium hydroxide solution, pour the mixture into a flat dish and heat in the oven at 130°C. The saccharated hydroxide gradually dissolves to an almost black solution. If to a sample of this solution (enough to colour 10 ml. of distilled water red-brown) dilute hydrochloric acid is added drop by drop it precipitates when pH 8 is reached. With continued heating, the pH at which this precipitation takes place falls gradually from 8 to 3. When the required precipitation point is reached (the original specimen used clinically with success had pH 5.7) the temperature of the oven is reduced to 90°C., the product is evaporated to dryness, and dissolved in 500 ml. of distilled water to give a solution containing 2 per cent. of elemental iron. After filtering through a Whatman No. 50 filter paper, and autoclaving, it is ready for use. Variations in the method of preparation are discussed and an account given of toxicity tests with these preparations leading to the selection of the best samples for intravenous administration. The macroscopical and microscopical findings in mice receiving lethal doses are described and the manner in which saccharated iron oxide produces

its toxic effects is discussed. Until the method of preparation is sufficiently reliable to ensure the constant production of satisfactory samples the authors consider that biological standardisation is necessary.

S. L. W

PHARMACOGNOSY

Indian Belladonna, Studies on. R. Chatterjee and J. K. Lahiri. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 11.) Indian belladonna, *Atropa acuminata* Royle, was compared morphologically with *A. belladonna* Linn. and found to differ mainly in the structure of the leaves. As a result of a critical study of specimens of *Atropa* from the Calcutta Herbarium, the authors suggested that the Indian species should be reduced to a variety of the type species and henceforward be called *A. belladonna* Linn. var. *acuminata* (Royle) R. Chatterjee and J. K. Lahiri. *A. lutescens* Jacquemont was said to be synonymous with *A. belladonna* Linn. var. *acuminata* (Royle), hence the statement by Chopra that *A. lutescens* (which is of low alkaloidal content) is used as an adulterant to Indian belladonna seemed without foundation. The hyoscyamine content of the roots from Indian sources is fairly high; commercially the roots are used for the preparation of atropine, and can be used for hyoscine since this constitutes about 15 per cent. of the total alkaloids.

G. R. K.

***Juniperus occidentalis*, Hooker, Sierra Juniper Wood.** E. F. Kurth and H. B. Lackey. (*J. Amer. chem. Soc.*, 1948, 70, 2206.) Trees of this species grown in Oregon were collected, the bark removed, and representative specimens of sapwood, heart-wood, whole wood, stumpwood and root-wood obtained. After room-drying to a moisture content of less than 10 per cent. the various samples were extracted with ethyl ether; the extractive ranged from 2.96 per cent. (stumpwood) to 6.50 per cent. (heart-wood). Approximately 1 per cent. of additional material, soluble in acetone, was chiefly a catechol phlobaphene. The ether extract was soluble in light petroleum to an extent of 2.53 per cent. on the weight of the sap-wood and 3.01 per cent. on the weight of the whole wood. The light petroleum extract consisted of resin acids, oleic acid and high molecular weight tactonic acids, a mixture of α - and β -sitosterol, and a hydroxyresene $C_{18}H_{31}O$. The volatile oil from the trunk of the tree ranged from 0.9 to 1.25 per cent. and appeared to consist of cedrol; the oil obtained from the rootwood contained cedrene and cedrol.

R. E. S.

***Remijia pedunculata*, Observations on the Bark of.** H. W. Youngken. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 27.) Studies of the physical characteristics and histology of *Remijia pedunculata* bark, a recognised source of quinidine sulphate in the U.S.P. XIII, and of quinine during the war, are reported. The materials and methods used in these studies are outlined. The unground bark is described and shown to differ from cinchona in having brittle cork which readily separates from the bark and is therefore absent or partly absent from most commercial specimens. A detailed description of the histology of the stem bark is given and differences between it and cinchona bark noted, a striking difference being the absence of microcrystals. New additional anatomical data are also reported and the powdered bark is described. Illustrations of pieces of the unground drug, the cross bark and the diagnostic tissue elements of the powdered

PHARMACY

GALENICAL PHARMACY

Cetyltrimethylammonium Bromide, Efficacy of in Ointment Bases. L. P. Prusak and A. M. Mattocks. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 67.) Six ointments comprising 1 per cent. of cetyltrimethylammonium bromide in hydrophilic ointment U.S.P., hydrophilic petrolatum U.S.P., simple ointment U.S.P., tragacanth glycerite N.F., pectin paste N.F., and a carbowax ointment base prepared by mixing carbowax 1500 with 10 per cent. of water, were added in amounts of 0.5 g. to 5 ml. quantities of nutrient broth inoculated with *Staphylococcus aureus*, and the mixtures were incubated at 37°C. for 3- and 24-hour periods. One loopful (0.05 ml.) of each mixture was sub-cultured in a medium containing lecithin, which inhibits the action of the bactericide, incubated for 48 hours and the tubes were read for turbidity. Control tests were carried out to determine the extent to which the ointment bases interfered with the action of the bactericide. Pectin paste and carbowax ointment base proved to be suitable ointment bases. The ointments prepared with hydrophilic ointment and hydrophilic petrolatum had no bactericidal activity, while those prepared with simple ointment and tragacanth glycerite released such small amounts of bactericide as to be practically ineffective. G. R. K.

Saccharated Iron Oxide for Intravenous Administration. J. H. Nissim and J. M. Robson. (*Lancet*, 1949, 256, 686.) Samples of saccharated iron oxide differ widely in toxicity owing to differing methods of preparation. The following method permits of better control and provides material of lower toxicity. Dissolve 25 g. of anhydrous sodium carbonate in 1 l. of distilled water and add 50 g. of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$). Carbon dioxide is evolved and the ferric hydroxide formed dissolves in the excess of ferric chloride to give a dark purple solution. Add sodium carbonate solution gradually to reprecipitate all the iron as ferric hydroxide, and adjust to pH 7. Wash away the sodium chloride formed by repeated additions of distilled water in quantities of 1000 to 2000 ml., allowing the ferric hydroxide to settle and decanting, continuing until the supernatant fluid acquires a brownish tinge. Add 166 g. of sucrose, followed by 30 ml. of 15 per cent. sodium hydroxide solution, pour the mixture into a flat dish and heat in the oven at 130°C. The saccharated hydroxide gradually dissolves to an almost black solution. If to a sample of this solution (enough to colour 10 ml. of distilled water red-brown) dilute hydrochloric acid is added drop by drop it precipitates when pH 8 is reached. With continued heating, the pH at which this precipitation takes place falls gradually from 8 to 3. When the required precipitation point is reached (the original specimen used clinically with success had pH 5.7) the temperature of the oven is reduced to 90°C., the product is evaporated to dryness, and dissolved in 500 ml. of distilled water to give a solution containing 2 per cent. of elemental iron. After filtering through a Whatman No. 50 filter paper, and autoclaving, it is ready for use. Variations in the method of preparation are discussed and an account given of toxicity tests with these preparations leading to the selection of the best samples for intravenous administration. The macroscopical and microscopical findings in mice receiving lethal doses are described and the manner in which saccharated iron oxide produces

its toxic effects is discussed. Until the method of preparation is sufficiently reliable to ensure the constant production of satisfactory samples the authors consider that biological standardisation is necessary.

S. L. W.

PHARMACOGNOSY

Indian Belladonna, Studies on. R. Chatterjee and J. K. Lahiri. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 11.) Indian belladonna, *Atropa acuminata* Royle, was compared morphologically with *A. belladonna* Linn. and found to differ mainly in the structure of the leaves. As a result of a critical study of specimens of *Atropa* from the Calcutta Herbarium, the authors suggested that the Indian species should be reduced to a variety of the type species and henceforward be called *A. belladonna* Linn. var. *acuminata* (Royle) R. Chatterjee and J. K. Lahiri. *A. lutescens* Jacquemont was said to be synonymous with *A. belladonna* Linn. var. *acuminata* (Royle), hence the statement by Chopra that *A. lutescens* (which is of low alkaloidal content) is used as an adulterant to Indian belladonna seemed without foundation. The hyoscyamine content of the roots from Indian sources is fairly high; commercially the roots are used for the preparation of atropine, and can be used for hyoscyne since this constitutes about 15 per cent. of the total alkaloids.

G. R. K.

***Juniperus occidentalis*, Hooker, Sierra Juniper Wood.** E. F. Kurth and H. B. Lackey. (*J. Amer. chem. Soc.*, 1948, 70, 2206.) Trees of this species grown in Oregon were collected, the bark removed, and representative specimens of sapwood, heart-wood, whole wood, stumpwood and root-wood obtained. After room-drying to a moisture content of less than 10 per cent. the various samples were extracted with ethyl ether; the extractive ranged from 2.96 per cent. (stumpwood) to 6.50 per cent. (heart-wood). Approximately 1 per cent. of additional material, soluble in acetone, was chiefly a catechol phlobaphene. The ether extract was soluble in light petroleum to an extent of 2.53 per cent. on the weight of the sap-wood and 3.01 per cent. on the weight of the whole wood. The light petroleum extract consisted of resin acids, oleic acid and high molecular weight lactic acids, a mixture of α - and β -sitosterol, and a hydroxyresene $C_{18}H_{31}O$. The volatile oil from the trunk of the tree ranged from 0.9 to 1.25 per cent. and appeared to consist of cedrol; the oil obtained from the rootwood contained cedrene and cedrol.

R. E. S.

***Remijia pedunculata*, Observations on the Bark of.** H. W. Youngken. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 27.) Studies of the physical characteristics and histology of *Remijia pedunculata* bark, a recognised source of quinidine sulphate in the U.S.P. XIII, and of quinine during the war, are reported. The materials and methods used in these studies are outlined. The unground bark is described and shown to differ from cinchona in having brittle cork which readily separates from the bark and is therefore absent or partly absent from most commercial specimens. A detailed description of the histology of the stem bark is given and differences between it and cinchona bark noted, a striking difference being the absence of microcrystals. New additional anatomical data are also reported and the powdered bark is described. Illustrations of pieces of the unground drug, the cross section of the bark and the diagnostic tissue elements of the powdered

ABSTRACTS

bark are included. *Remijia pedunculata* bark is shown to satisfy the identification test for cinchona in the N.F. VIII; hence it is suggested that this test be deleted in the N.F. IX or made more specific for cinchona. G. R. K.

***Opuntia vulgaris*, a new Source of Pectin.** H. Diacon and V. Massa. (*Ann. pharm. Franc.*, 1949, 6, 457.) *Opuntia vulgaris* Mill., which is common in Tunisia, gives a good yield of pectin. From 2 kg. of the fresh twigs, 14 g. of calcium magnesium pectate were prepared. The anti-hæmorrhagic action of this material was determined, and it was concluded that the action was superior to that of the pectin used by previous authors. This superiority, it is considered, is due solely to the high content of calcium and magnesium.

G. M.

PHARMACOLOGY AND THERAPEUTICS

Amethocaine Hydrochloride. Severe Toxic Effects when used for Bronchoscopy. C. A. Jackson. (*Brit. med. J.*, 1949, 1, 99.) The occurrence of toxic reactions in 2 patients treated with amethocaine to secure local analgesia before bronchoscopy is reported. The procedure adopted was to give 2 lozenges of benzocaine 200 mg. to suck 40 minutes pre-operatively followed after 10 minutes by 11 to 16 mg. of morphine. In the anaesthetic room, the fauces, the posterior pharyngeal wall and both pyriform fossæ were painted with less than 2 ml. of a 2 per cent. solution of amethocaine hydrochloride containing adrenaline 1 in 5000, and finally 2 ml. of the same solution was injected between the cords. Soon after injection, both patients became unconscious and had convulsions. In one patient, endotracheal oxygen and carbon dioxide and venepuncture effected recovery, but in the second, who had received 4 ml. by injection, deep cyanosis supervened and the pulse stopped, necessitating cardiac massage. Although the total period of cardiac arrest was about 4 minutes and unconsciousness persisted for 4 days, complete recovery took place. To avoid the occurrence of severe reactions the following precautions are suggested: (a) a barbiturate should be given by mouth pre-operatively; (b) an amethocaine pastille should be sucked 30 minutes before examination; (c) the total dose of amethocaine should not exceed 80 mg., and the solution should contain adrenaline, 1 in 5000; (d) application by spray should not be used; (e) amethocaine should not be applied to inflamed, traumatised or highly vascular surfaces (especially in the urethra), and (f) it should not be used for allergic, severely debilitated or cachectic patients. Treatment of toxic reactions should include artificial respiration with oxygen-carbon-dioxide mixture, using an endotracheal tube if necessary, intravenous administration of a rapidly-acting barbiturate to control the convulsions, and administration of respiratory and cardiac stimulants.

G. R. K.

Antabuse, Preliminary Report on Clinical Trials. R. G. Bell and H. W. Smith. (*Canad. med. Ass. J.*, 1949, 60, 286.) Antabuse is the Danish trade name for tetraethylthiuramdisulphide. Patients receiving this drug have an abnormal reaction to alcohol in the body, though the drug itself has few effects when administered in daily doses of 0.5 g. over a period of several months. The symptoms produced by alcohol after administration of antabuse are probably due to interference with the oxidation of alcohol so that abnormally high levels of acetaldehyde are produced in the body.

This report is mainly concerned with the symptoms produced when alcohol is taken after antabuse. 5 to 10 minutes after ingestion of a moderate amount of alcohol (20 g. of ethyl alcohol) there is a sensation of heat in the face, accompanied by flushing of the face and upper part of the body, with throbbing of the head and neck and accelerated pulse. After larger doses of alcohol (40 to 50 g. of ethyl alcohol) nausea may begin 30 to 60 minutes after the cardiovascular symptoms and may result in copious vomiting. A considerable fall in blood pressure may occur. The duration of the symptoms may last from half an hour to several hours but after a few hours sleep the patient feels completely well again. Antabuse will prove a valuable adjunct in the treatment of the alcoholic patient.

S. L. W.

Curare; A Method of Assay Using Rats. M. G. Allmark and W. M. Bachinski. (*J. Amer. pharm., Ass., Sci. Ed.*, 1949, 38, 43.) A unit consisting of 15 separate compartments floored partly with wire netting and partly with galvanised iron and fitted with a cover to keep the rats in their respective compartments was attached to a frame at a 60° angle. Commercial samples of intocostin and *d*-tubocurarine chloride were used for the tests, diluted with water to a concentration required to produce responses when injected subcutaneously into each rat. After injection, each rat was placed in a separate compartment and observed for 20 minutes; if it fell off the wire netting within this time it was considered a reactor. It was found that very few rats fell off after 20 minutes. To test the validity of the method several three-dose assays were carried out using 15 rats on each dose. *d*-Tubocurariné chloride was used as a standard for each assay. Results were tabulated and the methods of Bliss were followed in making the calculations. The slopes of the regression lines were found not to differ significantly for the 10 assays reported and in no assay did the slope of the regression line for intocostin differ from that of *d*-tubocurarine chloride, nor could it be determined that the weighted means of the slopes differed, indicating that the responses in rats are the same for both products. Further tests of accuracy were recorded using solutions of known potency. The mean actual error for these assays was 5.7 per cent. Changing the slope of the frame to 75° did not increase the accuracy of the method. It compared favourably with the rabbit head-drop cross-over test and the mouse method.

G. R. K.

Decamethonium Iodide (C10) in Anaesthesia. G. Organe. (*Lancet*, 1949, 256, 773.) The use of decamethonium iodide in 150 operations of many different types, have established that this drug is a safe and satisfactory substitute for *d*-tubocurariné and that it may be used in unselected cases. It is roughly five times as potent as *d*-tubocurarine chloride but has a shorter effect, and it produces a neuromuscular block which is not affected by anticholinesterases. A single intravenous injection of 3 mg. in light surgical anaesthesia produces in most patients good muscular relaxation without unduly depressing respiration. Its action is relatively evanescent and further injections are made at intervals of 10 to 40 minutes as required. The dose depends on the preceding interval—after 40 minutes a further 3 mg. will probably be necessary. Pentamethonium iodide in a dose 10 times that of decamethonium iodide is an effective antidote. Thoracic and abdominal breathing fail, and recover, together. It seems to act similarly to *d*-tubocurarine in reducing laryngeal muscular irritability. There appears to be no

direct effect on the cardiovascular system, even with relatively large doses. Post-operative vomiting occurs in less than 25 per cent. of patients, post-operative collapse seems considerably less frequent than after *d*-tubocurarine chloride, and there have been no cardiovascular complications. Urinary retention lasting for 24 hours occurred in 9 per cent. of patients. All the common anæsthetic agents have been used with no obvious difference in effect, and a mixture of decamethonium iodide 4 mg. with thiopentone 1 g. has been used successfully.

S. L. W.

Dextran as a Plasma Substitute. G. Thorsén. (*Lancet*, 1949, 256, 132.) A proprietary form of dextran is described as a 6 per cent. solution of a polydispersoid glyucose-polymer dextran, in which most of the molecules have been hydrolytically given a molecular weight conforming to that of an albumin, with 0.9 per cent. of sodium chloride added. Its viscosity lies between that of blood and plasma, and its specific gravity somewhat exceeds that of human plasma. It is non-toxic and does not injure the tissues either locally or systematically. After an intravenous injection of 1 or 2 l. of dextran the plasma-dextran level rises to 1 to 2.5 g./100 ml., and after an initial fall due to elimination of a low molecular fraction through the kidneys it falls at an even rate. During the initial renal excretion, when about a quarter of the dextran given is excreted, the urine-dextran level rises to 7 g./100 ml. without sign of renal injury. After that no dextran can be detected in the urine, the remainder of the dextran of a higher molecular weight being presumably metabolised. In Sweden today the hospital transfusion services rely to a large extent on dextran for emergency cases. It has been given to 5000 patients, and as much as 4 l. has been given in a single infusion. Very good results have been obtained both in the treatment and prevention of shock, and it is stated to be as good as plasma in shock from burns. Its use has not been found to affect fertility, foetal development or growth.

S. L. W.

Dimethyl Ether of *d*-Tubocurarine Iodide, Pharmacology of. H. O. J. Collier, S. K. Paris and L. I. Woolf. (*Nature*, 1948, 161, 817.) The dimethyl ether of *d*-tubocurarine iodide was compared with *d*-tubocurarine in the following ways: (1) in the intact mouse, rat and rabbit; (2) in a preparation of the rat under nembutal anæsthesia, similar to that of Raventos, in which the contractions of the rectus femoris muscle, in response to repeated condenser charges applied to its motor nerve, are recorded on smoked paper; (3) in the rat phrenic nerve-diaphragm preparation *in vitro*. In the intact rabbit the intensity of action of the dimethyl ether compound was shown to be many times greater than that of *d*-tubocurarine, while the slopes of the two duration curves were closely similar, indicating that the two substances are removed from their site of action at similar rates. The dimethyl ether compound, therefore, has a greater specific action at the myoneural junction in this species; this is again shown in the rat, though to a somewhat less extent than in the rabbit, by the *in vitro* experiments on the phrenic nerve-diaphragm preparation. In the intact mouse, the myoneural junction is slightly less sensitive to the dimethyl ether compound than to *d*-tubocurarine, but the rate of elimination of the former is slightly less than that of the latter. In the rat, *in vivo*, the duration of action of the dimethyl ether compound is many times greater than that of *d*-tubocurarine, whereas it has only about three times the intensity of action, which indicates that it is removed much more slowly from its site of action in this species. In the

Raventos preparation of the rat, the weight of the dimethyl ether compound required to reduce the tension of the muscular response to a given extent was one-third to one-half that of *d*-tubocurarine, when each drug was administered by jugular cannula, and the reduction lasts considerably longer. Ligation of the renal arteries and veins in the preparation prior to administration of either substance increases duration of action of each but does not prevent recovery of the muscular contraction. The dibenzyl and di-isopropyl ethers of *d*-tubocurarine iodide were prepared. The dibenzyl compound exhibited in the mouse about one-third the activity of *d*-tubocurarine and about one-half the intensity of action in the rat and the rabbit; it did not exhibit any more prolonged action. The dimethyl ether of *l*-bebeerine di-methochloride was also prepared. The ED50 and LD50 of this compound were found to be about twice those of *d*-tubocurarine. It was three times as potent as *d*-tubocurarine in reducing tension of muscular contraction in a Raventos preparation, and the effect lasted four times as long.

S. L. W.

Local Anæsthetics, a Comparison of. H. S. Hamilton, B. A. Westfall and J. K. W. Ferguson. (*J. Pharmacol.*, 1948, 94, 299.) A new series of indices, to be called Relative Ratings (R.R.) is proposed for the comparison of potency and toxicity of local anæsthetics in relation to cocaine or procaine. For each the drug of reference should be named, e.g., the Relative Rating with reference to cocaine should be designated R.R. (cocaine). In addition, the bases of comparison should be specified in tables or in the text. Example for any drug X:

$$\frac{\text{LD50 of cocaine}}{\text{LD50 of X}} = \text{Relative Toxicity (cocaine) or Toxicity relative to cocaine.}$$

$$\frac{\text{EC50 of cocaine}}{\text{EC50 of X}} = \text{Relative Potency (cocaine) or Potency relative to cocaine.}$$

Then:

$$\frac{\text{Relative Potency (cocaine)}}{\text{Relative Toxicity (cocaine)}} = \text{Relative Rating (cocaine).}$$

LD50 values (by intraperitoneal injections in mice), tissue toxicities (by intradermal injections in guinea-pigs), and EC50 values for infiltration anæsthesia (by intradermal injections in guinea-pigs), and surface anæsthesia (of the guinea-pig cornea) were determined for procaine, metycaine, monocaine, naphthocaine, butacaine, octocaine, cocaine, amethocaine (pontocaine) and cinchocaine (nupercaine). The LD50 values decreased in magnitude in the order given. Adrenaline hydrochloride in solution with each anæsthetic increased the systemic toxicity of procaine and metycaine and decreased the toxicity of amethocaine and cinchocaine significantly, but had no effect on the toxicity of the other drugs. From these determinations Relative Rating indices for the 9 drugs are defined and estimated by the authors for infiltration anæsthesia, localised block anæsthesia, and corneal anæsthesia. The two relatively new drugs naphthocaine (β -diethylaminoethyl-4-amino-1-naphthoic acid) and octocaine (2-(1-methyl-heptyl)-2-dimethylethyl *p*-amino-benzoate) have high Relative Ratings for all four types of anæsthesia.

S. L. W.

Orthoxine, Pharmacology of. B. E. Graham and M. H. Kuizenga. (*J. Pharmacol.*, 1948, 94, 150.) In a study of a large series of phenyl propylamines, for the purpose of obtaining compounds more active than ephedrine

as bronchodilators and possessing little or no pressor activity, the methoxy-phenylisopropylamines appeared most interesting. The intravenous toxicities, pressor actions and bronchodilator properties of 14 of these amines are recorded. *ortho*-Methoxy- β -phenylisopropyl methylamine hydrochloride (Orthoxine), because of its high activity and low toxicity, was investigated further. Perfusion experiments with constrictor agents on isolated lungs showed it to be a much more effective agent than ephedrine for relieving bronchoconstriction. Using isolated strips of jejunum or ileum, it was 4 to 8 times as effective as ephedrine in relieving intestinal smooth-muscle spasms induced by histamine, acetylcholine and barium chloride, and 5 times as effective in quieting the normal contractions of unstimulated intestinal muscle. On the non-pregnant uterus it was only half as active as ephedrine in stimulating the muscle to contraction. It produces little or no pressor response, and 4 times as much orthoxine as ephedrine must be administered to normal dogs to produce the bradycardia characteristic of the latter. Intestinal smooth-muscle tests indicate that it possesses anti-histaminic properties of the order of 1/20 that of benadryl but much greater than that of ephedrine. Its toxicity, chronic and acute (except for higher intravenous toxicity), is of the same order as that of ephedrine. The authors suggest that the drug may be of value in the treatment of asthma, and clinical trials are being carried out.

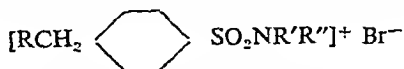
S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

Dithiocarbamic Acid Derivatives: Action Against Human Pathogens. C. R. Miller and W. O. Elson. (*J. Bact.*, 1949, 57, 47.) The activity of compounds in this group against plant pathogens prompted the investigation of their *in vitro* activity against human bacterial and fungal infections. The substances tested included series of dithiocarbamates, thiuram monosulphides and thiuram disulphides, and a number of miscellaneous sulphur-containing compounds. The most active antibacterial compounds studied were tetramethylthiuram disulphide and sodium dimethyldithiocarbamate, their activity being greatest against *Streptococcus pyogenes*, much less against *Str. faecalis*, *S. aureus* and *Escherichia coli*, and weakest against *Pseudomonas aeruginosa*. The same compounds were also the most active against the fungi tested, *Trichophyton gypseum* being most affected, *Epidermophyton floccosum*, *Microsporium canis*, *Sporotrichum schenckii*, *Blastomyces dermatitidis* much less, and *Candida albicans* least.

H. T. B.

Quaternary Ammonium Sulphonamides, Antibacterial Actions of. C. A. Lawrence and G. R. Goetchius. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 424.) Six members of a series of compounds of the general structure



have been examined for antibacterial action against a number of organisms. The two most active compounds were those in which R = tetradecyldimethylamino-, R' = R'' = H, and R = pyridine, R' = dodecyl, R'' = H. These two compounds were highly active against both gram-positive and gram-negative organisms but not against *Pseudomonas aeruginosa*. The antibacterial actions of these compounds are due to the ammonium component in the molecule and not to the

BOOK REVIEWS

PHARMAKOLOGISCHE METHODEN by L. Ther. Pp. 443 and Index. Wissenschaftliche Verlagsgesellschaft m.b.H. Stuttgart, 1949.

This book, a product of post-war Germany, should be of interest to those pharmacologists, physiologists, and others concerned with the techniques of experimental investigation of the properties of medicinal substances. The volume has many good features to recommend it as a practical book of reference, or a guide book to laboratory procedures, not least of which is the presence of some 244 line drawings of apparatus or techniques, and the citing of over 1,200 references to original literature describing technical procedures. The apparatus depicted is somewhat out of date, many of the blocks dating from the time when the favourite mode of heating depicted was a small Bunsen flame. The text deals mostly with continental methods with a preponderance of Teutonic origins. It is of interest that nothing much more recent than a decade ago is described. The descriptions apply briefly to a multiplicity of methods rather than provide an adequate description of any one procedure. The chapter on testing of vermicides is interesting, that on striped muscle singularly inadequate. The others vary in quality. There is no evidence of acquaintance with any of the recent advances in physics, biochemistry or operative procedures in this volume. The chapter on the handling of common laboratory animals is useful, though the rhesus monkey might have been included with advantage. The type and paper are good, the binding of a lower standard. The book might be described as a useful guide to the more classical methods of investigation of drug action, with a particular interest for the teacher of pharmacology on account of the description of several useful methods of demonstrating the action of drugs on tissues.

J. D. P. GRAHAM.

A TEXT BOOK OF PHARMACOGNOSY, by G. E. Trease. Pp. VIII-811 and Index. Fifth Edition. Bailliere, Tindall and Cox, London, 1949, 30s. net.

A perusal of this text-book shows the wide field covered by modern pharmacognosy. Besides descriptions of a large number of vegetable and animal drugs, information is given on such varied materials as cotton, silk and surgical dressings, bacteria and fungi (including *Penicillium* spp. and yeast), chalk and kieselguhr, shellac, gelatin, beeswax and spermaceti, wool alcohols and gums; in short, the raw materials from the vegetable and animal kingdoms which go to furnish the pharmacist with his dressings, his vaccines and antibiotics, his plant insecticides and cosmetic creams, as well as the usual tinctures, infusions and tablets, pure alkaloids and crystalline products like picrotoxin and tubocurarine. The arrangement of the information is similar to that of the previous edition, the bulk of the book consisting of descriptions of crude drugs, etc., arranged in order of Phyla and families. Many of the descriptions include microscopical characters.

Besides this descriptive part there are short chapters on the history, commerce, cultivation, storage and evaluation of crude drugs. There are also chapters of a general nature on microscopical technique, constituents and extraction of crude drugs, and analysis (including fluorescence and chromatographic analysis). This information is necessary in order that the practitioner may be able to apply modern technique both to the description and evaluation of crude drugs.

As the author's aim is to cover the requirements of examination syllabuses

one should not perhaps expect much information on more recent work in pharmacognosy and additions to the materia medica such as alginates, *Ammi Visnaga*, the *Holarrhena* alkaloids, *Erythrina* alkaloids and rutin, but one would expect information on thyroid and pancreas, both of which are in the new syllabus for the degree of Bachelor of Pharmacy. Apart from these omissions the author's aim is well fulfilled in this book.

J. W. FAIRBAIRN.

PRECIS DE CHIMIE TOXICOLOGIQUE, by F. Schoofs. 2nd edition, 1948. Pp. 509 and Index. Les Presses Universitaires de Liege, Maison des Etudiants, Liege.

The scope of toxicology, and of toxicological chemistry in particular, is so wide that it is in the light of the author's statements that any volume on this subject must be considered. In the preface to the first edition it is stated that the book is intended for students as an introduction to the fundamental ideas of toxicological chemistry, not as an encyclopædic treatise but as a guide to those poisons which are more frequently encountered. As regards practical details it is said that these are given to enable "the more important methods used for the detection and determination of poisons" to be performed. It must be stated at the outset that, although the volume forms a useful introduction to the general principles of toxicological chemistry and deals with a wide range of poisons it would, in the opinion of the reviewer, be of limited value to anyone confronted with the actual task of toxicological analysis.

The work is divided into eight chapters: (1) general discussion; (2) gaseous substances; (3) volatile poisons and poisons isolated by distillation; (4) acids; (5) poisons extracted by immiscible solvents; (6) metallic poisons; (7) non-metallic poisons; (8) the purity of reagents. The first chapter on generalities is, to a chemist, one of the most useful in the book. It includes a discussion of the various routes of absorption and excretion of poisons, of the relation between molecular structure and toxicity, and contains some notes on the general precautions to be taken in toxicological analysis. It is to be regretted that the volume does not contain more references to the original literature. Most of the references are to text-books and little is given of recent analytical procedures and in particular of methods capable of detecting and estimating small quantities of organic and inorganic poisons. In dealing with lead, for example, useful notes are given on acute and chronic poisoning, on the toxic dose and on the method of elimination. It is difficult, however, to appreciate the value of the method given for the detection and estimation of lead when one considers that following this the use of a dithizone solution in chloroform is dismissed with the sentence "Cette solution verte, agitée avec un sel de plomb dissous dans l'acétate d'ammonium en présence de cyanure de potassium, donne une coloration rouge." Some sections, such as that on alcohol, are well written and fairly comprehensive, although that on barbituric acid derivatives occupies only six pages, a small number in view of the present day prevalence of this type of poisoning. The book is bound in paper covers and almost all of the pages require cutting. The editing and proof reading have been well done, although the formula for D.D.T. (p.396) is wrongly given. It is likely that this volume will be mostly useful in providing an account of the pharmacological action and general chemistry of a fairly wide range of poisons.

NEW REMEDIES

Dolcin* tablets contain calcium succinate 2.8 gr., and acetylsalicylic acid 3.7 gr. and are advocated for use in the treatment of rheumatic conditions. This succinate-salicylate therapy is based on the theory that the widespread systemic disturbance seen in some forms of arthritis is due to an alteration in tissue metabolism and respiration. Calcium succinate counteracts these effects by stimulating oxygen utilisation by the tissues; it also averts the depressant effect of salicylates on blood prothrombin. Dolcin is indicated in the treatment of rheumatic fever; articular rheumatism, including rheumatoid arthritis and osteoarthritis; non-articular rheumatism, including fibrositis, neuritis, lumbago and sciatica; arthritis associated with the menopause and gout. The initial dosage is 3 tablets four times daily, reduced to 2 tablets four times daily when the acute symptoms have subsided. The tablets are usually well tolerated and side-effects are rare. The tablets are issued in bottles of 100.

S. L. W.

Folybden* Tablets each contain 1.7 mg. of folic acid and 3 gr. of molybdenised ferric sulphate. The addition to the iron of traces of molybdenum is stated to produce an increased rate of hæmoglobin formation. The tablets are suggested for use in the treatment of refractory normocytic anæmias when satisfactory hæmoglobin regeneration is not produced by the administration of liver extract or of folic acid alone. The dosage is 1 tablet three times daily after meals.

S. L. W.

Genabrom* is a sedative preparation, one teaspoonful containing $7\frac{1}{2}$ gr. of sodium bromide in concentrated yeast extract. When stirred with hot water it forms a palatable soup-like beverage. It is indicated in nervous conditions, such as hysteria, anxiety states, irritability, nervous vomiting, travel sickness, neurasthenia, and wherever a sedative is required. For insomnia, one or more small teaspoonfuls are dissolved in a cupful of warm water before retiring. Large doses may be given in epilepsy to lessen the frequency and severity of attacks. It is issued in jars containing 45 and 90 g.

S. L. W.

Lantigen* is a desiccated bacterial vaccine for oral use containing antigenic substances prepared from a selected group of freshly isolated bacterial species chosen for their strong antigenic properties. The vaccine is presented as a slightly opalescent colloidal solution, containing as preservatives 0.1 per cent. of phenol and 0.01 per cent. of mercurithiosalicylate. It is claimed that the vaccines are absorbed by the alimentary mucosa and that their action is not impaired by digestion, as antibodies are produced before digestion begins. The vaccines should be taken daily, and the dose should be retained in the mouth as long as possible before swallowing, since the immediate local immunising response obtained is of additional benefit. Six Lantigen vaccines are prepared as follows:—"A," for coryza; "B," for nasopharyngeal catarrh and chronic bronchitis; "C," for rheumatic disorders of pathogenic origin; "D," for staphylococcal skin infections; "E," for hay fever and asthma; "F," for whooping cough.

S. L. W.

Magsilate* tablets contain acetylsalicylic acid 5 gr., magnesium trisilicate $1\frac{1}{2}$ gr., magnesium hydroxide $1\frac{1}{2}$ gr., sugar and flavouring q.s. The aspirin is protected by a coating of sugar and flavouring agent, which, in turn, is

surrounded by layers of magnesium trisilicate, magnesium hydroxide, and sugar. The tablets are not intended to be swallowed but to be eaten like a sweet. The advantages claimed for this method of presentation are the absence of free salicylic acid, freedom from irritation of the gastric mucosa, and convenience of administration. Cartons are issued containing 12 tablets.

S. L. W.

Metheph* is a proprietary brand of methylephedrine hydrochloride suitable for oral administration. It is claimed to have the following advantages over ephedrine: (1) the blood pressor action is approximately only one-tenth that of ephedrine; (2) it does not stimulate the central nervous system; (3) it has a persistent slowing and deepening effect on respiration, and there is no secondary acceleration as with ephedrine. It is indicated for the relief of the bronchial spasm of asthma, especially in hypertensive asthmatics, the average dose being 1 tablet three times daily, with 1 or 2 tablets at bedtime. Clinical trials have also indicated its value in the treatment of enuresis in children. It is supplied in tablets of 2/3 gr. in bottles of 25, 100 and 500.

S. L. W.

Tineafax* is a compound undecylenate ointment for the treatment of fungus infections of the foot. Undecylenic acid is not only effective against dermatophytic fungi but it possesses the blandness essential for prolonged prophylaxis and treatment, and being related to the normal constituents of sweat it does not cause irritation of the skin. It is also active over a wide pH range and is resistant to dilution by bathing and perspiration. In addition to the zinc salt of undecylenic acid, the ointment contains the fungicide zinc naphthenate, mesulphen, which possesses keratolytic properties, methyl salicylate, terpineol, and a small quantity of phenylmercuric acetate as preservative. Tineafax Powder, which is used in conjunction with the ointment, contains 10 per cent. of undecylenic acid, as the potassium salt, in an inert base. The ointment is applied night and morning, the powder being dusted on after the ointment has been rubbed in. The powder may also be used prophylactically by dusting into stockings, socks and shoes. The majority of cases of infection are cleared by this treatment in 7 to 21 days. The ointment is supplied in 1 oz. collapsible tubes, and the powder in 2 oz. tins.

S. L. W.

Trimetron: A New Antihistaminic Drug. I. W. Schiller and F. C. Lowell. (*New Eng. J. Med.*, 1949, 240, 215.) Trimetron, 1-phenyl-1-(2-pyridyl)-3-dimethylaminopropane, has been shown in animal experiments to combine high antihistaminic activity with low toxicity. The authors now report the results of clinical tests on 84 allergic patients of either sex, ranging in age from 5 to 60 years. Doses of 25 to 125 mg. were given orally for several weeks. All of 12 patients with urticaria obtained satisfactory relief. Of 55 patients with perennial allergic rhinitis satisfactory or partial relief was reported by 47; the 3 reporting partial relief were not improved by increasing the dosage; 13 of 15 hay fever patients also obtained satisfactory or partial relief. Side reactions, chiefly drowsiness and dryness of the mouth, were mild; they occurred in only 10 patients. The drug is considered to be particularly useful in the treatment of perennial allergic rhinitis.

H. T. B.

REVIEW ARTICLE

ANALGESICS : SOME DEVELOPMENTS

BY A. D. MACDONALD, M.D., M.A., M.Sc.

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INTRODUCTION

AT a time which Dale has called the golden age of therapeutics, when chemotherapy is making one spectacular advance after another, it is still important to remember how much of medicine is concerned with the treatment of symptoms. The relief of pain will of course follow the cure of the disease, but this may be slow while the pain is urgent and the ætiology still obscure. Analgesics may be defined as drugs which reduce or relieve the sensation of pain without producing loss of consciousness or parallel depression of other senses. Thus general anæsthetics, while used at times, as in labour, for escape from pain are not true analgesics nor are they desirable in the everyday treatment of pain.

Local anæsthetics are also now of established value in the treatment of such localised trouble as fibrositis, and skilled and experienced practitioners can often give remarkable and sustained relief by such injections. There has been some use of local anæsthetics intravenously in the relief of pain. This is difficult to justify pharmacologically, for these drugs, apart from cocaine, which is manifestly unsuitable for such application, are rapidly cleared from the blood-stream and fail to follow the Hughlings Jackson "law" in their action. Instead of the usual descending paralysis produced by narcotics—descending in the sense that the most recently developed and highly specialised functions of the central nervous system are the first to be depressed—intravenous local anæsthetics are quite liable to paralyse respiration in doses which scarcely affect spinal reflexes. It is true that analgesics similarly break the "law." Adrian¹, while doubting whether pain is as much appreciated at the level of the thalamus as Head maintained, agrees that since . . . "Pain needs no learning to increase its potency. This must be due to a direct effect on the basal ganglia." Analgesics should therefore act at this level as on the cerebral cortex, but while there is presumptive evidence of selective effects, there is need for something like tracer technique to establish real localisation of drug action. Probably by introducing a radioactive isotope into the analgesic molecule, the concentration of the drug could be estimated in the tissues even in great dilution, and its fate followed.

Methods of testing the potency of analgesic drugs are numerous. Where long series of related compounds have to be compared, some animal screening is first necessary. The promising drugs may then be assessed comparatively on human volunteers, as in a recent paper by Prescott *et al*². Probably even such results in man require checking by

experience in the actual relief of human suffering before much weight can be attached to them.

Screening tests have been carried out on all sorts of laboratory animals, and a variety of painful stimuli have been tried of which heat in one form or another has been most popular because of the precision with which it can be repeated and measured. It is important that the intensity and duration of the stimuli should be such as to cause no tissue damage, since such would inevitably lead to changes in threshold. Fortunately there is no need for stimuli of such severity. In assessing possible analgesic value the toxicity of the proposed remedy and any side-actions which it may elicit must be taken into account, but it may not be possible to appreciate such considerations till clinical trials are instituted. In man, heat may be applied, till pain is felt, by the use of a resistance coil or by focusing the light of a powerful lamp on a fixed area of blackened skin. Other methods preferred by some workers for the study of analgesic action consist of assessments of the modification of the pain which the drug affords when pain is elicited by injections of hypertonic saline or by muscular contraction in an ischæmic limb. These and other methods are listed with numerous references in the recent chemical review of the synthetic analgesics by Bergel and Morrison³. There is abundant evidence that there is liable to be a large psychological element and a substantial individual variation in all such assessments, so that rigorous controls are necessary.

While analgesics have been shown to be very active when applied to the mid-brain directly, in quite small doses, the precise mechanism of their action is still unknown. Unlike most narcotics, analgesics have little effect on the oxygen consumption of the brain slice and little effect on choline-esterase systems, but they may block the availability of amino acids or other essential metabolites to certain nerve-cells.

OPIATES

Opium has been used in the relief of suffering for at least three thousand years. It is nearly 150 years since morphine was isolated from opium. Yet as recently as 1938 Fournau⁴ claimed that morphine and a few of its derivatives could alone be considered true analgesics. The coal-tar antipyretics, widely used for certain nerve and muscle pains, seemed so far behind opium in the relief of pain associated with organic disease that Fournau suggested they be called "antalgics," while he called cannabis and the related tetrahydro-cannabinols "euphorigenics," *euphoria being the most striking part of the effects they normally produce*. The hemp drugs have a definite analgesic action in animals but, rather curiously, increase in dosage does not enhance this analgesic effect. (Davies, Raventos and Walpole⁵.) They have a long history in therapeutics, and the synthetic compounds may bring them again to the fore (Macdonald⁶, Avison, Morrison and Parkes⁷). While cannabis is scheduled with the dangerous drugs and is known to be a frequent drug of addiction it is claimed to be free from any such risk in therapeutics, but its applications there have been so wide that one hesitates

to accept them. Its value in the amelioration of mood in mental disorders appears to be established.

The literature of the opiates is enormous, and no attempt to review it can be included here. The United States Public Health Services cover it in two large volumes (Kreuger, Eddy and Sumwalt⁸). In experimental animals, the action of opium and of total opium alkaloids is substantially the action of the morphine they contain. Potentiation by the other alkaloids is hard to demonstrate, and so is potentiation by neostigmine, though this has been claimed. Such use of a choline-esterase inactivator is the more revolutionary in that for many years it has been customary to give atropine or hyoscine with morphine to reduce its side-actions if not to enhance its analgesic effects. Many experienced physicians use morphine almost exclusively.

Many workers have published tables in which the analgesic activity of a series of drugs is assessed numerically in terms of morphine. Such tables may be misleading. Here let it be stated that the ideal analgesic is not established by a claim that it is, say, six times stronger than morphine. Morphine is usually strong enough. What is wanted is a drug which has morphine's anxiety- and pain-relieving qualities together with less or none of its undesirable side actions. Morphine lies open to criticism in that it is liable :—

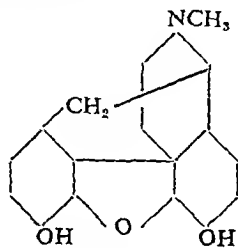
- (1) to depress respiration.
- (2) to produce nausea and vomiting.
- (3) to be constipating.
- (4) to produce tolerance and the chance of addiction.
- (5) to increase itching and skin irritation.
- (6) to be dangerous in susceptible subjects and young children.

A substance like codeine (methymorphine) though it has only $\frac{1}{6}$ or less of the analgesic power of morphine, is often preferred because of its relative freedom from these side-actions. Diamorphine (diacetylmorphine) on the other hand, though a powerful and reliable analgesic, leads to habit formation so frequently and so quickly that its manufacture and importation are forbidden in the United States. There is a considerable movement to ban it similarly here, because of recent evidence of increased consumption and increasing addiction in various other countries. Mono-acetylmorphine is about four times as active as morphine when assessed by the methods of Hardy and Wolff (irradiation of blackened skin) or Smirk and Alam (pain produced by exercise of ischæmic limb), yet produces only "a moderate euphoria," much less nausea and vomiting, and allows of increased voluntary muscular effort in the presence of severe pain.

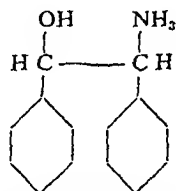
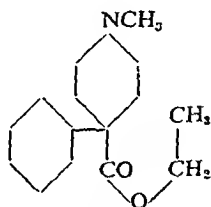
Dihydromorphinone (Dilaudid), and methyl dihydromorphinone (Metopon), are in the diamorphine class or better as regards relieving pain. They are effective in the late stages of malignant disease, and good by mouth, but their actions are short and they may certainly provoke addiction.

The attempts to modify the structure of morphine in such a way as to relieve it of its side-actions but retain its analgesic value cannot be

said to be strikingly successful though much research has been directed to this problem. Where the chemist has been successful in increasing the analgesic action by his manipulations, his product has usually shown increased toxicity, though not always a parallel increase in the two actions. As a rule the increase in potency is accompanied by a decrease in duration of action. Many regard the search for an analgesic which is not a potential drug of addiction as futile, but there is a sustained effort to find a morphine substitute which is less depressant to the respiratory centre. The failure so far to synthesise morphine in spite of attempts by many distinguished chemists has probably been a major difficulty but Gulland and Robinson⁹ here and Grewe¹⁰ in Germany have made marked progress. There is an alternative method of investigation—to try and identify the basic part of the morphine molecule with which analgesia is associated and then test various chemical modifications of this for potency.



Morphine.

 β -Hydroxy- α ; β -diphenylethylamine.

Pethidine.

This is the technique so successfully followed by Dodds¹¹ in the development of the synthetic œstrogens. In the case of morphine he believed that diphenylethylamine was the core of its efficiency, and tested 18 compounds by the Hardy-Wolff and other techniques. Peak activity was found in hydroxydiphenylethylamine, which gave "complete relief in doses of 200 to 400 mg. four hourly." At first this looked promising but later it appeared that many forms of pain were uninfluenced by these drugs though in malignant disease they were claimed to be of special value. In comparative tests in animals they show no significant activity.

PETHIDINE

Pethidine was introduced (Eisleb and Schaumann¹²), not as an analgesic but as a spasmolytic. Its original name was Dolantin, and it was introduced here as Dolantal, in America as Demerol. Its ability to

relieve pain was discovered later, and its rather remote chemical relationship to morphine suggested. Though as an analgesic it seemed to be in the codeine rather than in the morphine class, (Woolfe and Macdonald¹⁴), the facts that it had any real pain-depressing activity and that it provided a convenient molecule for modification by the synthetic chemist gave a fresh impetus to research in analgesia. Pethidine was the best of Eisleb's compounds as assessed by Schaumann¹³. A long series of related compounds synthesised by Bergel and his team were assessed pharmacologically by Macdonald and Woolfe¹⁵. Some had a longer action on mice than pethidine and some had a slightly stronger action—notably 2'-methylpethidine—but although certain of these derivatives received some clinical encouragement (Glazebrook and Brantwood¹⁶) the differences were not sufficiently great to be very important. Since then an ethyl ketone, Hoechst No. 10720 (ketobemidone) has been claimed to be ten times as active as pethidine, and has had a promising clinical trial (Kirchhof¹⁷).

Pethidine is of established value in relieving the pain of labour, and this may be related to its additional action as a spasmolytic. When combined with gas and air in doses of 100 mg, to 200 mg., however, it is reported to double the incidence of asphyxia neonatorum and the same risk is recorded on a much larger series when used with trichloroethylene¹⁸.

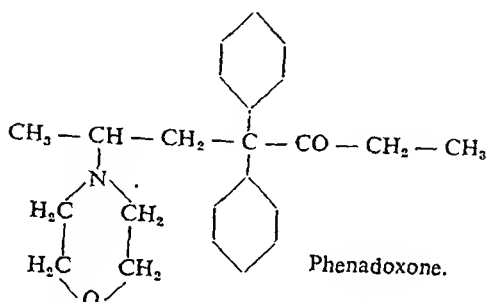
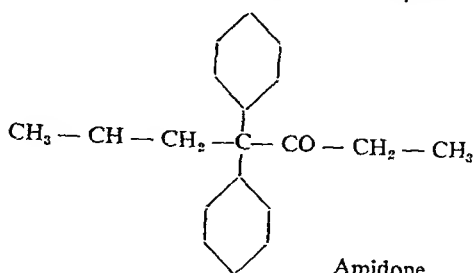
Is it therefore concluded that the routine use of pethidine by midwives cannot be approved. Whether the disadvantages of pethidine can be avoided in the new compound ketobemidone (Hoechst 10720) or in some of the heptanones or hexanones is still uncertain. The demand for a safe and reliable analgesic for use in the conduct of labour is insistent, and the use of inhalers for nitrous oxide or trilene without premedication seems to be reasonably satisfactory in some eighty per cent. of cases. This problem is now receiving widespread attention.

AMIDONE AND RELATED COMPOUNDS

Amidone (Hoechst 10820) also known here as miadone and physeptone, and in America as methadon, adanon and dolophine, was revealed during the investigation of I. G. Farbenindustrie¹⁹ at the end of the war. This compound again stimulated a fresh outburst of research. It was found to be at least as powerful an analgesic as morphine, yet less hypnotic. Its use however is often complicated by prolonged nausea and vomiting, and today it may be more important as a source of new compounds which may retain analgesic efficacy yet be free from these unpleasant side actions. Of such *isoamidone*, which was discarded by the original Hoechst workers has had favourable reports both here and in America^{2,20} and 2-dimethylamino-5-acetoxy-4:4-diphenylheptane and the 2-morpholinopropyl compound (C.B. 11) are relatively free from unpleasant toxic effects.

It is a pity to find the latter (Phenadoxone, Heptalgin,) advertised as "activity six times that of morphine" even if certain animal tests confer such a ratio. It is much more important to be assured that the acute

toxicity is relatively much lower and that side effects apart from mild drowsiness with full dosage are negligible. Relative freedom from serious respiratory depression and constipating action is freedom indeed.



Wilson and Hunter²¹ comparing amidone, phenadoxone, and pethidine found that 5 mg. of amidone only relieved ischæmic pain in six of ten subjects, whereas 5 mg. of phenadoxone relieved nine of the ten. Both were better than 50 mg. of pethidine, but this was strikingly more euphorigenic than the newer drugs.

The optical isomers of amidone have been prepared (Thorp *et al.*²²) and compared with the racemic forms (Thorp²³). The lævo-isomer is responsible for the effects of amidone on the central nervous system, while the dextro compound shares equally in the spasmolytic, local anæsthetic and toxic actions on the circulation (but not on the respiration) in experimental animals.

No important recent developments have taken place in Fourneau's "antalgics." Amidopyrine is probably still the most potent but has lost favour because of its occasional effects on the bone-marrow. Aspirin is still the most widely used, yet phenacetin is regarded by critical observers as a more effective drug, though little used by itself. These two, in combination with a little codeine, at present enjoy an enormous vogue but there is some doubt whether the claimed "potentiation" in such mixtures will bear pharmacological scrutiny.

The barbiturates which were at one time claimed to be analgesic as well as hypnotic have failed to live up to any such claim except in anæsthetic doses—the use of the shorter-acting compounds as intravenous anæsthetics is undoubtedly a major advance. The barbiturate-antalgic combination, so bitterly opposed in the past by Willcox, has been

restricted by the inclusion of barbiturates in Schedule IV of the Poisons Act and by the wide publicity given to the toxic risks of amidopyrine which is similarly scheduled. Wayne²⁴ has recently emphasised the risks involved in the abuse of such drugs.

Most new analgesics, whether related to morphine or not, have been introduced as "free from morphine's tendencies to produce tolerance and addiction." None has seriously stood up to critical tests of such claims—perhaps it is too much to expect of an analgesic. But the advances in this field in the past ten years are full of hope and promise, and whether an approximately ideal drug will be provided by the acetylated alcohols corresponding to the ketones of the amidone group, by some other derivative or in some quite different way, it will surely be found in due course.

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RESEARCH PAPERS

THE ANTIDOTAL ACTIVITY OF SOME DITHIOLS AND ACETYLDITHIOLS IN MICE POISONED WITH OXOPHENARSINE

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It is well known that dimercaprol (2:3-dimercaptopropanol, BAL) and other dithiols reduce the toxicity of compounds of arsenic to animals and enzyme systems (Peters, Stocken and Thompson¹; Stocken and Thompson²; Fitzhugh, Woodard, Braun, Lusky and Calvery³; *et al.*). A number of 1:2-dithiols and three $\alpha:\omega$ -dithiols not previously studied have been examined for toxicity and anti-arsenical activity, and the results are reported here. Some of the dithiols were prepared from the corresponding acetylated dithiols, and as it seemed possible that deacetylation might occur in the body, the activity of the acetylated dithiols was also examined. In order to have an adequate basis for this study, the activity of dimercaprol and dimercaprol glucoside (BAL-Intrav) was first explored in some detail.

The compound of dimercaprol and oxophenarsine is more toxic than oxophenarsine alone (Peters and Stocken⁴; Friedheim and Vogel⁵), and the toxicity of this compound has been examined further. Its high toxicity suggests that circumstances may exist in which dimercaprol potentiates oxophenarsine poisoning. This possibility has been examined, as has the possibility of potentiation by other dithiols.

METHODS

Mice weighing 17 to 25 g. were used. They were kept at a temperature between 20° and 24°C. during and after experiments. They were fed on a standard diet of cubes of the following composition:—wheat bran, 19.2 per cent.; wheat ground middlings, 19.2 per cent.; Sussex ground oats, 19.2 per cent.; ground maize, 9.5 per cent.; ground barley, 9.5 per cent.; meat and bone meal, 9.5 per cent.; skim milk powder, 7.0 per cent.; fish meal, 4.8 per cent.; dried yeast, 1.3 per cent.; cod-liver oil 0.4 per cent.; salt mixture, 0.4 per cent. (manufacturer's figures). Food was withdrawn on the evening before experiments, and was restored immediately after treatment. Except where it is otherwise indicated, all poisons were injected into the muscles of the right hind leg, and all thiols into the muscles of the left hind leg. Doses were adjusted according to body-weight, and have been expressed in mg.-molecules/kg. (mM/kg.) to facilitate comparison between the amounts of poison and antidote used. The mice were observed at least 1, 2, 4, 8, 20 to 21, 28 to 32

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and 48 hours after injection in most experiments, and approximate estimates of the survival time were made from these observations. Deaths were rare after 48 hours, and were not included in estimates of the mean survival time or of the mortality. The mice were watched until four weeks after the day of injection. With one exception discussed below, late deaths occurred seldom and erratically, and have not been reported as their significance is doubtful. Post-mortem examinations and weighing of organs, when performed, were carried out within an hour of death. The preparation and chemical properties of the dithiols and acetylated dithiols used (Table V) have been or will be reported elsewhere (Evans and Owen⁶; Evans, Fraser and Owen⁷). Some of the free dithiols (dimer-caprol glucoside, 0:16, 0:17, 0:19, 0:20 and 0:24) were prepared from their barium salts, and the solutions so obtained were standardised by titration in acid solutions at 0°C. with iodine (Weatherall and Weatherall⁸, and *infra*). The other substances were received pure or nearly pure and were generally used freshly dissolved or suspended in olive oil or peanut oil. Dithiodulcitol was dissolved in aqueous sodium hydroxide, and the solutions were neutralised with boric acid. Certain acetylated dithiols (0:11, 0:14) which were difficult to dissolve in oil or in propylene glycol were dissolved in diethylene dioxide and the volumes injected were kept below 1.0 ml./kg. Otherwise the volumes injected were 10.0 ml./kg. in toxicity tests and 5.0 ml./kg. each of dithiol and arsenical in antidotal tests. The doses reported have been corrected for purity. The organic solvents in the volumes used were not lethal and had no detectable anti-arsenical activity.

RESULTS

I The toxicity of oxophenarsine.

The LD₅₀ of oxophenarsine by subcutaneous injection in mice is given as 0.164 mM/kg. by Ercoli and Wilson⁹. Cranston, Clark and Strakosch¹⁰ give the value 0.17 mM/kg. for intraperitoneal injection and cite the manufacturer's figures of 0.1 mM/kg. for intravenous and about 0.12 to 0.13 mM/kg. for subcutaneous injection. Eagle, Hogan, Doak and Steinman¹¹ found an LD₅₀ of 0.165 mM/kg. intraperitoneally. Data obtained in the present series of experiments are collected in Table I and show that the mortality after intramuscular injection varied over the range 0.08 to 0.20 mM/kg. with an LD₅₀ of about 0.14 mM/kg. In two experiments in which oxophenarsine was injected intraperitoneally in some mice and intramuscularly in others there was no significant difference in the mortality on any dose.

Death occurred more rapidly when the doses of oxophenarsine were large, and even with the rather crude measurements of survival time, in the range studied the means of the logarithms of the survival times were approximately linearly related to the logarithm of the dose in groups in which there were no survivors (Fig. 1 and Table I). When some animals survived, the mean survival time tended to be less than the value expected for a mortality of 100 per cent. An arbitrary correction for survivors

might be used, but it is doubtful whether such a procedure would be helpful in interpreting the results.

The weight of the lungs was measured in some of these mice. The lungs were substantially heavier than normal in mice poisoned with a small dose of oxophenarsine, and only slightly heavier when the dose was large (Table I). As the mice poisoned with a small dose lived longer, it seems probable that the development of heavy lungs depended

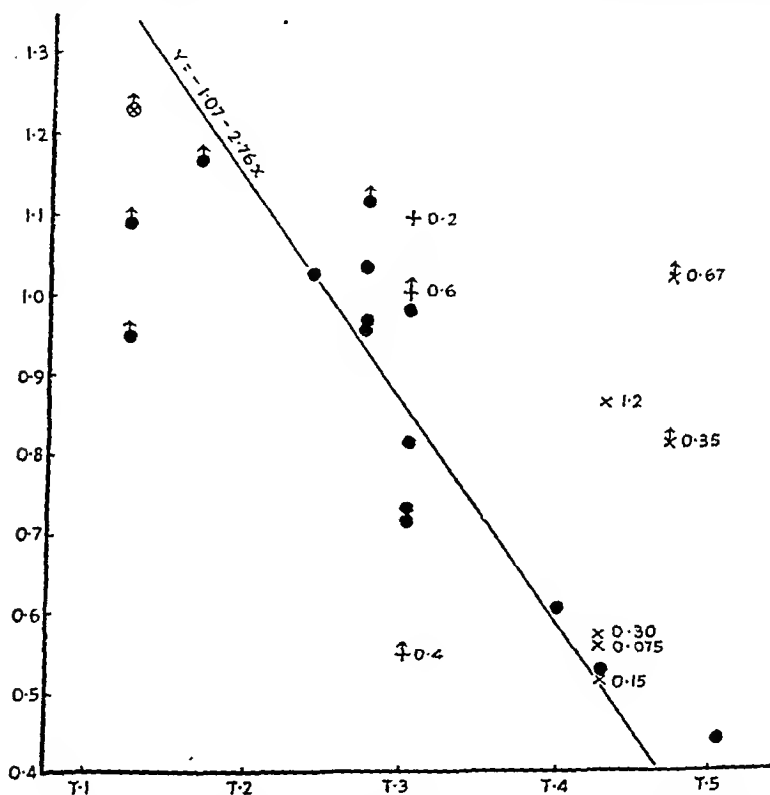


FIG. 1. The relation between log dose and log survival time in mice poisoned with oxophenarsine.

Ordinates—Survival time in log. hours.

Abscissae—Dose of oxophenarsine in log. mM/kg.

Each point represents a group of 5 to 10 mice, all of which died. Each arrow represents a group of 5 to 10 mice of which at least half died. No allowance has been made for survivors in calculating the mean survival time.

- No treatment
 - + Treated with dimercaprol
 - × Treated with dimercaprol glucoside
 - ⊗ Injected with an approximately equimolar mixture of oxophenarsine and dimercaprol glucoside
- } The figure against the point gives the dose of dithiol in mM/kg.

The regression line is that which fits best the points for oxophenarsine alone ($Y = -1.07 - 2.76X$).

The doses of dimercaprol glucoside are approximate.

ANTIDOTAL ACTIVITY OF DITHIOLS AND ACETYLDITHIOLS

TABLE I

THE TOXICITY OF OXOPHENARSINE GIVEN BY INTRAMUSCULAR INJECTION IN MICE

Dose of oxophenarsine	Total mortality No. dying/total No.	Log survival time of mice dying		Lung weight of mice dying	
		Mortality	Log Hours M±S.E.	Number of Mice	mg./g. body wt. M±S.E.
mM/kg.	per cent.				
0	Normal Mice*	—	—	10	6.6±0.32
0.067	0/15	—	—	—	—
0.080	4/9	4/9	1.25±0.19	2	12.2±2.46
0.093	1/5	—	—	—	—
0.107	2/8	—	—	—	—
0.113	4/19	3/10	0.94	—	—
0.120	6/21	5/15	1.05±0.15	4	10.0
0.133	26/48	7/10	1.09±0.10	—	—
		16/20	0.95±0.07	—	—
		2/8	1.32	—	—
0.147	15/30	4/10	0.94±0.10	4	8.2±0.66
		8/10	1.17±0.10	—	—
0.153	8/20	5/10	1.23±0.13	—	—
0.160	15/18	—	—	—	—
0.173	15/17	10/10	1.03±0.08	—	—
0.178	6/15	5/10	0.95±0.11	—	—
0.187	38/43	6/6	0.96±0.10	—	—
		9/9	0.97±0.16	—	—
		10/10	1.04±0.09	10	6.6±0.42
		8/10	1.12±0.10	—	—
0.200	80/82	10/10	0.82±0.12	9	7.9±0.39
		10/10	1.00±0.09	8	7.1±0.37
		8/8	0.72±0.18	5	7.9±0.41
		10/10	0.73±0.16	—	—
0.250	34/34	10/10	0.61±0.10	—	—
0.267	16/16	10/10	0.53±0.03	—	—
0.320	10/10	10/10	0.44±0.01	—	—

* Killed by breaking neck.

on the length of life, but conclusive evidence on this point was not obtained.

II. The toxicity of the oxophenarsine-dimercaprol compound.

As was shown by Peters and Stocken⁴, when oxophenarsine and dimercaprol are mixed in equimolar amounts before injection or given as the previously crystallised compound, the toxicity is substantially enhanced. This has been confirmed for the pure compound given by intramuscular injection (Table II), but it is interesting to note that in rats and mice it is not true when the compound is given intraperitoneally. In these circumstances the compound is actually less toxic than oxophenarsine, the toxicity of which is about the same by either route. After intramuscular injection of just lethal amounts of the compound, the mice died if anything more rapidly than mice poisoned with a (rather larger) just lethal amount of oxophenarsine. Their viscera were unusually congested and their lungs hæmorrhagic and substantially heavier than normal. After intraperitoneal injection some mice had convulsions and died in a few minutes: others survived for a few hours and at death tended to show less pulmonary congestion. Rats showed the same series of symptoms and difference in toxicity by intramuscular and intraperitoneal injection, and again the toxicity of oxophenarsine by either route was intermediate. Deaths and survivals observed after different dosages, in all in a dozen rats, do not suggest that the toxicity in rats is greatly different from that in mice.

III. The effect of dimercaprol in oxophenarsine poisoning.

The mortalities when various doses of dimercaprol have been given at various times after various doses of oxophenarsine are shown in Table III. In Figure 2 the dose of oxophenarsine has been plotted against the

TABLE II
THE TOXICITY OF THE OXOPHENARSINE-DIMERCAPROL COMPOUND AND OF MIXTURES OF OXOPHENARSINE AND DIMERCAPROL GLUCOSIDE IN MICE

Substance	Dose mM/kg.	Route of administra- tion	Mortality No. dying/total No.	Log survival time of mice dying Log hours M±S.E.	Lung weight of mice dying	
					No. of Mice	mg./g. M±S.E.
Oxophenarsine, dimercaprol compound	0.013	Intra- muscular	0/5	—	—	—
	0.027		0/5	—	—	
	0.040		2/20	—	2	14.2
	0.053		2/5	—	—	—
	0.067		16/20	0.87±0.049	14	14.0±0.80
	0.107		4/5	—	—	—
	LD50=0.055 mM/kg.					
	0.013	Intra- peritoneal	0/5	—	—	—
	0.027		0/5	—	—	—
	0.053		0/5	—	—	—
	0.107		0/5	—	—	—
	0.120		1/5	—	1	11.6
	0.213		0/5	—	—	—
	0.233		2/9	—	2	14.7
	0.300		5/9	0.50±0.186	5	12.0
	LD50=0.295 mM/kg.					
Oxophenarsine D.G. Mixture approx 3:1	0.133*	Intra- muscular	16/20	1.15±0.08	7	8.1±0.59
Oxophenarsine D.G. Mixture approx. 3:4	0.133*	"	11/20	1.23±0.09	7	8.5±0.31
Oxophenarsine alone	0.133	"	16/20	0.95±0.07	6	7.7±0.53

* mM/kg. of oxophenarsine in mixture.

dose of dimercaprol with different symbols for mortalities below and above 50 per cent. A line has been drawn to separate the two sets of points, and therefore gives approximately the LD50 of combinations of the two substances. As the LD50 of dimercaprol alone is of the order of 1.0 mM/kg., 0.60 mM/kg. approaches the largest dose which can be used therapeutically without itself causing death. This dose is quite effective against 0.30 mM/kg. of oxophenarsine, and still has some effect even when it is given 80 minutes after a smaller dose of the poison; but these are about the limits of its activity. These findings are in reasonable agreement with those of Ercoli and Wilson⁹ under slightly different conditions. Mice which were treated with dimercaprol but did not survive generally lived longer than untreated poisoned mice. This point has been explored further, as reported elsewhere (Weatherall and Weatherall⁵), and slight but just significant acceleration of death has been observed when very small doses of dimercaprol were given to mice

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poisoned with very large doses of oxophenarsine. With equivalent quantities of poison and antidote, or an excess of antidote, only prolongation of life has been observed.

Observations have not been made on the mortality from sublethal doses of oxophenarsine treated with less than one equivalent of dimercaprol, but no potentiation was observed by one equivalent (Table III). Poisoned

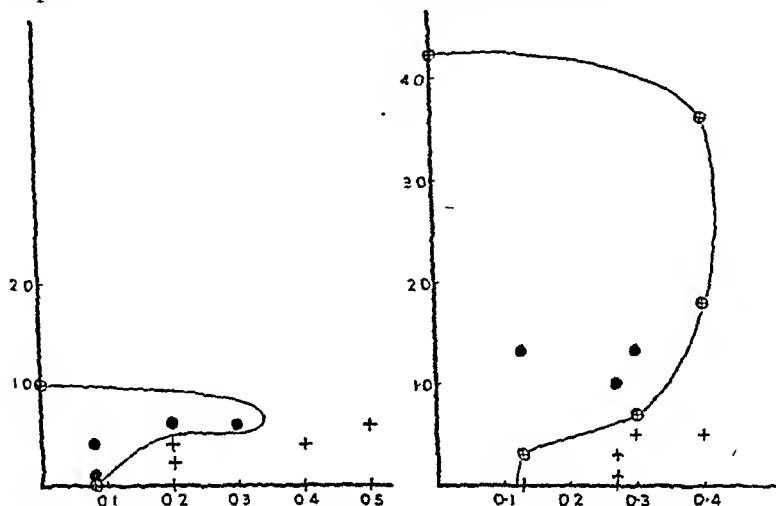


FIG. 2. Isobols for oxophenarsine and dimercaprol (left-hand graph) and for oxophenarsine and dimercaprol glucoside (right-hand graph).

Ordinates—Dose of dithiol in mM/kg.

Abscissae—Dose of oxophenarsine in mM/kg

Each point represents the mortality for a group of mice.

- Less than 50 per cent. mortality.
- ⊕ Approximately 50 per cent. mortality.
- + More than 50 per cent. mortality.

— Line separating points where the mortality is less than and more than 50 per cent.

mice treated with dimercaprol had heavier lungs than had untreated mice poisoned with the same dose of oxophenarsine. Dimercaprol itself increases the lung weight slightly, and this increase may be an additive effect. The lungs were not heavier than those of untreated mice poisoned with less oxophenarsine and living about as long as the mice treated with dimercaprol: so if the increase in lung weight depends on the time of exposure rather than directly on the dose of oxophenarsine, the increase is not appreciably affected by dimercaprol. In any case, the great increase in lung weight observed in poisoning with oxophenarsine-dimercaprol was not observed when the two substances were given separately, and attempts to imitate its toxicity in this way have been practically unsuccessful.

IV. The toxicity of dimercaprol glucoside and its effects in oxophenarsine poisoning.

As dimercaprol glucoside has not been purified and isolated and the only materials available for biological work have been solutions pre-

TABLE III

THE MORTALITY OF MICE POISONED WITH OXOPHENARSINE AND TREATED WITH DIMERCAPROL OR DIMERCAPROL GLUCOSIDE

Treatment, minutes after oxophenarsine		Immediate							Im- med.	20 minutes	30 minutes	40 minutes	80 minutes	160 minutes
Dose of oxophenarsine mM/kg.		0.08	0.13	0.20	0.27	0.30	0.40	0.50						
No treatment
Dimercaprol :—
0.08 mM/kg.	...	4/9	—	8/8	—	—	10/10	10/10	—	—	5/5	—	—	—
0.20 "	...	2/9	—	8/8	—	—	—	—	—	—	—	—	—	—
0.40 "	...	0/9	—	6/9	—	—	10/10	—	—	—	—	—	—	—
0.60 "	...	—	—	1/8	—	2/9	—	10/10	3/5	2/5	—	2/5	4/5	5/5
No treatment
Dimercaprol glucoside :—
ca 0.08 mM/kg.	...	—	24/30	—	24/24	—	—	—	—	—	10/10	—	—	—
ca 0.30 "	...	—	10/20	—	10/10	—	—	—	—	—	—	—	—	—
ca 0.50 "	...	—	—	—	10/10	9/10	10/10	—	—	—	—	—	—	—
ca 0.70 "	...	—	—	—	—	4/9	—	—	—	—	—	—	—	—
ca 1.00 "	...	—	—	—	11/24	—	—	—	—	—	—	—	—	—
ca 1.33 "	...	—	7/20	—	—	0/10	—	—	—	—	—	—	—	—
ca 1.80 "	...	—	—	—	—	—	—	—	0/10	2/10	6/9*	4/5	10/10	10/10
ca 3.60 "	...	—	—	—	—	—	—	—	—	—	—	—	—	—

* Dose of oxophenarsine 0.33 mM/kg.

pared from the barium salt, itself of uncertain purity and always with less than the theoretical thiol content, figures for toxicity are necessarily approximations and depend on what criterion is used in calculating the concentration of the solution used. Danielli, Danielli, Mitchell, Owen and Shaw²² and Danielli *et al.*²³ give figures based on the weight of the barium salt used per 100 ml. of solution prepared, and allow only for the replacement of the barium atom by two hydrogen atoms. In the present series of experiments it has been found that the toxicity of different batches, measured in these units, varies very widely, and that more constant values are obtained when the solutions are standardised by iodine titration at the time the toxicity is determined and the doses are measured in accordance with the titres. Iodine titration at room temperature gives a rapidly fading and rather arbitrary end-point, and better results are obtained in N hydrochloric acid at 0°C. This was not at first appreciated, and in early experiments solutions were standardised by a modification of Sampey and Reid's method¹⁴ for monothiols, by adding excess of iodine, leaving overnight, and estimating the excess with sodium thiosulphate. This procedure gave a better defined end-point than direct titration at room temperature, but it subsequently appeared that with dithiols in these conditions the reaction proceeded beyond the disulphide stage, to a variable extent according to the amount of iodine present. In most of the indirect titrations, the excess of iodine was within the limits of 50 to 100 per cent. excess, and when further titrations were carried out under these conditions in parallel with direct titrations in N hydrochloric acid at 0°C., the indirect titre varied only between 2.5 and 3.5 times the direct titre. When only an indirect titration was performed and the excess of iodine was not outside the above limits, the strengths of solutions have been calculated on the assumption that the direct titre would have been one-third of the indirect, and are regarded as rough approximations. It has been assumed throughout that, in the direct titration, one gramme-molecule of iodine is equivalent to one gramme-molecule of dimercaprol glucoside.

Data about various batches of dimercaprol glucoside are given in Table IV, where the doses are expressed (A) as derived from the amount of barium salt used in making the solution and (B) from iodine titration. The dose-mortality curve was determined in one experiment with a large number of mice, and as the estimated slope of the line relating log dose to probit mortality was in good agreement with less accurate estimates obtained in other experiments, the LD₅₀ was sometimes deduced from the mortality on a single dose by the use of this slope.

It will be seen that, whereas the most toxic solution (OB1) was about seven or eight times as lethal as the least toxic (142) when the concentrations were calculated from the amount of barium salt used, the difference was not more than two to threefold in terms of the iodine titre. If solutions prepared from specimen SO.1443 are not considered, the toxicity in terms of the iodine titre is reasonably constant. Specimens SO.1422 and SO.1443 were both somewhat unsatisfactory and de-

teriorated rapidly, possibly because they had not been freed sufficiently from traces of organic solvents (Owen, personal communication), and it seems reasonable to attribute the consistently higher toxicity of solutions prepared from SO.1443 to the presence of impurities. In the other solutions, toxicity is evidently attributable to the free thiol present, as might be expected; and the material which does not react with iodine, presumably chiefly products of oxidation, does not contribute much to the toxicity. The sulphhydryl content may also be assumed to regulate the anti-arsenical activity, and so all doses in the present work have been

TABLE IV
THE TOXICITY OF SOLUTIONS OF DIMERCAPROL GLUCOSIDE

Specimen of barium salt used in preparation	Soln. No.	Dose mM/kg. A	Mortality No. dying/total No.	Slope of line relating log dose and probit	LD50 mM/kg.	
					A	B
—	3	17.5 14.0 11.2	7/10 3/10 0/10	11.0	15.7	3.8
SO-1001 ... *	40	35.2 31.2 28.9	4/5 1/5 3/5			
SO-1001 ...	48	29.5	10/10		<29.5	<ca 3.5
SO-1229 ...	108	21.0 18.6 16.6 14.7 13.1 11.8 10.5 9.5	20/20 20/20 20/20 15/20 7/20 5/20 3/20 3/20	10.7	12.8	ca 4.4
SO-1422 ...	142	46.7	8/15		45.0	4.5
SO-1443 ...	OB1	10.5 7.9 5.2	8/8 8/8 2/16		5.9	2.1
SO-1443 ...	143	10.1	8/15	—	9.7	2.6
SO-1443 ...	144	23.8	5/15	—	25.6	2.1
9374A ...	241	14.0 12.6	14/15 9/15	12.5	11.9	—

(A) Calculated from the amount of barium salt used in preparing the solution.
(B) Calculated from the iodine titre of the solution (see text).

calculated from the results of iodine titration. As a result of doing so, a number of apparent irregularities in the behaviour of dimercaprol glucoside have disappeared. Another result of doing so is that the toxicity appears to be greater than the figures of Danielli *et al.*¹⁴ suggest. As their doses include in the weight material which does not behave as a free thiol, they give an exaggerated suggestion of the harmlessness of the substance. Nevertheless its toxicity is undoubtedly much less than that of dimercaprol.

The activity of dimercaprol glucoside against oxophenarsine is shown in Table III and Figures 1 and 2. The activity is on the whole similar to that of dimercaprol itself. If anything the glucoside is a little less

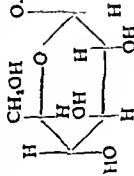
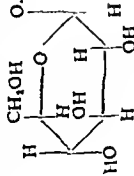
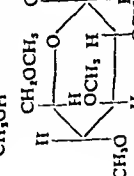
active when given at the same time as the oxophenarsine. About 0.8 mM/kg. of the glucoside saved 5 of 9 mice poisoned with 0.30 mM/kg. of oxophenarsine, while 0.6 mM/kg. of dimercaprol was sufficient to save 7 of 9: but the difference is not significant. As the glucoside was less toxic, more could be given, and it was consequently superior against very large doses of oxophenarsine. Its much greater activity in these conditions is strikingly illustrated by the much larger area enclosed by its isobol than by that of dimercaprol (Figure 2). On the other hand, once poisoning was established, the glucoside was less effective. Dimercaprol still saved an occasional animal 80 minutes after oxophenarsine had been given, whereas no reduction in mortality was obtained with the glucoside after 40 minutes, even with large doses and in rather more extensive trials. As Danielli *et al.* suggest, the water-soluble thiol probably enters cells less readily, and so has less access to arsenic once the arsenic has been fixed by the tissues.

Mixtures of oxophenarsine and dimercaprol glucoside in proportions about a 1 to 1 molar ratio have been injected intramuscularly in mice, and no enhancement of toxicity of the arsenical has been found (Table II). Deaths were neither increased nor accelerated, and the lungs were, as usual in mice poisoned with oxophenarsine and treated with a dithiol, only moderately heavier than normal. Solutions probably containing less than one molecule of dimercaprol glucoside per molecule of oxophenarsine gave a negative nitroprusside reaction, whereas the reaction was positive when the dithiol was in excess; so it may be assumed that the two substances had combined. The evidence does not suggest that the compound has any enhanced toxicity.

IV. The toxicity of other dithiols and their effects in oxophenarsine poisoning.

The results of toxicity tests are summarised in Table V. The figures are sometimes very approximate, either because the amount of the material available was limited and the toxicity was low, or because preliminary tests indicated that the toxicity was too high for the substance, however active as an antidote, to be likely to be of any therapeutic use. The LD₅₀s have been obtained either by injecting into one group of 15 to 20 mice a dose slightly larger than the LD₅₀ and into another group a dose slightly smaller and estimating the LD₅₀ by linear interpolation after transformation to log dose and probit mortality; or by the method of Kärber¹⁵. The efficacy against oxophenarsine was tested (a) by giving the antidote immediately after an LD₉₅ to 99 (0.16 to 0.20 mM/kg.) to show whether any protection at all was obtained; (b) by giving the antidote 40 minutes, and sometimes at other times, after the oxophenarsine, to show whether protection could be obtained late in acute poisoning; (c) by giving a 50 per cent. larger dose of oxophenarsine and the thiol immediately afterwards, to show whether the thiol was effective against massive poisoning. The dose of thiol chosen was about one-third of its LD₅₀, and tests (b) and (c) show roughly the maximal activity of dimercaprol and dimercaprol glucoside respectively at this level of

TABLE V
THE TOXICITY AND ANTI-ARSENICAL ACTIVITY OF SOME THIOLS AND ACETYLDITHIOLS

No.	Name	Formula	Mol. Wt.	Purity per cent.	LD50 mM/kg.	No. of mice in estimate of LD50	Activity against oxophenarsine	
							Immediate	After 40 minutes
0:13 0:17 0:24	Dimercaptol (BAL) 1:2-dimercaptobutane-3:4-diol 1:2-dimercaptopentane-3:4:5-triol 1:2-dimercaptotrioxane-3:4:5:6-tetrol	$\text{CH}_3\text{SH}\cdot\text{CHSH}\cdot\text{CH}_2\text{OH}$ $\text{CH}_3\text{SH}\cdot\text{CHSH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH}$ $\text{CH}_3\text{SH}\cdot\text{CHSH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH}$ $\text{CH}_3\text{SH}\cdot\text{CHSH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH}$	124 154 184 214	100 80 † †	1.0 1.0 1.7-2.5 1.7	99 20 28 29	††† ††† ††† †††	†† ††† ††† Not done
0:8 0:9	2:3-dihydroxypropyl 2:3-dimercapto- propyl ether 1:3-dihydroxy-2-propyl 2:3-dimer- captpropyl ether	$\text{CH}_2\text{OH}\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{O}\cdot\text{CH}_2\cdot\text{CHSH}\cdot\text{CH}_2\text{SH}$ $\text{CH}_2\text{OH}\cdot\text{CH}_2\cdot\text{O}\cdot\text{CH}_2\cdot\text{CHSH}\cdot\text{CH}_2\text{SH}$ 	198 198	78 92	1.3 1.3	55 50	††† ††	† †
—	(2:3-dimercapto-propyl) glucoside (di- mercaptol glucoside, BAL-Intrav)		286	†	3.8-4.7	230	††††	†
0:19 0:16 0:20	3(2':3'-dimercapto-propyl) mannitol... (2':3'-dimercapto-propyl) mannitol... (2':3'-dimercapto-propyl)sorbitol ...	$\text{CH}_2\text{OH}\cdot\text{CHOH}\cdot\text{CH}(\text{OCH}_2\text{CHSH}\cdot\text{CH}_2\text{SH})\cdot\text{CHOH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH}$ $\text{CH}_2\text{OH}\cdot\text{CHSH}\cdot\text{CH}_2\cdot\text{O}\cdot\text{CH}_2\cdot\text{CHOH}$ $\text{CH}_2\text{OH}\cdot\text{CHSH}\cdot\text{CH}_2\cdot\text{O}\cdot\text{CH}_2\cdot\text{CHOH}$	288 288 288	† † †	3.2 2.6 2.6	40 40 29	†††† †††† ††††	0 †† †††
0:23	2:3:4:6-tetramethyl 2':3'-dimer- captpropyl glucoside		342	94	5.0-8.0	9	†††	†

ANTIDOTAL ACTIVITY OF DITHIOLS AND ACETYLDITHIOLS

TABLE V--continued

No.	Name	Formula	Mol. Wt.	Purity per cent.	LD50 mM/kg.	No. of mice in estimate of LD50	Activity against oxophenarsine	
							Immediate	After 40 minutes
0:15	β mercapto-γ valerolthiolactone	$ \begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_2\text{SH}-\text{CH} \\ \quad \quad \\ \text{CO} \quad \quad \text{S} \\ \diagup \quad \diagdown \\ \text{CH}_2\text{SH} \end{array} $	148	100	0.9	40	† †	† †
0:22 0:12 0:1	1: 4-dithiothreitol 1: 4-dithioerythritol 1: 6-dithiodulcitol	$ \begin{array}{c} \text{CH}_2\text{SH} \quad \text{CH}(\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CO} \quad \quad \text{CO} \\ \diagup \quad \diagdown \\ \text{CH}_2\text{SH} \end{array} $	154 154 214	100 97 90	0.7 2.0-2.5 3.4, 2.2*	25 30 55	— † †	Not done 0
0:21 0:12 0:14 0:18	Diacetyl dimercaprol 3-acetoxy-1: 2-bisacetylthiopropene (triacetyl dimercaprol) 3: 4-diacetoxy-1: 2-bisacetylthiobutane 3: 4: 5-triacetoxy-1: 2-bisacetylthio- pentane	$ \begin{array}{c} \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \end{array} $	208 230 322 334	100 100 100 100	<2.5 1.6 3.0 >10.0	18 44 12 16	† † † † † † † †	Not done † Not done 0
0:5 0:6	2: 3-diacetoxypropyl 2: 3-bisacetyl- thiopropyl ether 1: 3-diacetoxy-2-propyl 2: 3-bisacetyl- thiopropyl ether	$ \begin{array}{c} \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \end{array} $	366 366	76 95	2.9 3.2	40 24	† † † † † †	Not done
0:11	Hexa-acetyl β-(2: 3-dimercaptopropyl) glucoside		538	100	>5.0	10	†	0
0:10 0:7 0:4	Diethyl 3: 4-bisacetylthiobutane-1: 1- dicarboxylate Ethyl 2: 3-bisacetylthiopropoxycetate Tetra-acetyl 1: 4-dithiothreitol	$ \begin{array}{c} \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \\ \quad \quad \\ \text{CH}_3\text{SAC}(\text{CH}_3\text{OH})\text{CH}_2\text{SH} \end{array} $	302 294 322	70 77 100	ca 10.0 2.8 >1.3	9 15 10	0 † —	0 Not done Not done
—	Cysteine	$ \begin{array}{c} \text{CH}_2\text{SH}-\text{CH}(\text{NH}_2)-\text{COOH} \end{array} $	121	100	—	—	†	0

— Accelerated death.
— Killed all mice on ca 0.67 LD50 of oxophenarsine.

†	Delayed death.	
†	Saved some lives against	ca. 1.5 LD50
††	Saved all lives against	ca. 1.5 LD50
†††	Saved all lives against	ca. 2.2 LD50

Ac * †
-CO.CH₃.
From mortality at 28 days.
† Prepared from barium salt : doses derived from iodine titre.

dosage. The quantitative method of assessing anti-arsenical activity by the increment produced in survival time (Weatherall and Weatherall⁸) had not been devised when the majority of these dithiols were tested, and so the foregoing procedure was used. Some of the substances were later evaluated by the more precise method, with results as reported in a separate paper and briefly indicated below.

Brief comments can be made on the individual substances.

A. 1:2-dithiols derived from polyhydric alcohols. The higher analogues of dimercaprol tended to be less toxic and less active against oxophenarsine. 1:2-dimercaptobutane-3:4-diol (0:13) was pharmacologically indistinguishable from dimercaprol. 1:2-dimercaptopentane-3:4:5-triol (0:17) was more water-soluble, less toxic, and less efficient when given late in oxophenarsine poisoning. 1:2-dimercaptohexane-3:4:5:6-tetrol (0:24) was about as toxic as the pentane derivative. It was not tested against oxophenarsine, but Weatherall and Weatherall⁸ found that it had no activity at all against phenylarsenoxide.

B. O-Ethers of dimercaprol. As with the dithiols derived from polyhydric alcohols, the more water-soluble substances with a longer carbon chain and more hydroxyl groups were less toxic, but here there was more loss of toxicity and less loss of therapeutic activity. The glucoside appeared to be the best of this group, but the sorbitol ether (0:20) was surprisingly active in established oxophenarsine poisoning and still gave 100 per cent. protection after 40 minutes. 1:3-dihydroxy-2-propyl 2:3-dimercaptopropyl ether (0:8) and the sugar ethers contain the grouping $-O-CH_2-CROH-CR_2OH$, to which Bradley and Berger¹⁰ attribute the ability of causing paralysis as does myanesin. Berger and Bradley¹¹ describe the paralysis as not accompanied by excitement, tremors, twitchings or convulsions at any time. None of these ethers produced exactly such a paralysis. An apparent flaccid paralysis was observed after nearly lethal doses of 6(2':3'-dimercaptopropyl) mannitol (0:16), but when disturbed the mice made quite powerful movements, and lethal doses of this substance, like most of the other dithiols, produced convulsions.

C. Other 1:2-dithiols. The γ -lactone of γ : δ -dimercaptovaleric acid (0:15) was a little more toxic than dimercaprol. Deaths occurred more quickly, usually within 90 minutes of injection, and were preceded by very vigorous convulsions. Congestion of the lungs was more conspicuous post-mortem than with most dithiols. Anti-arsenical activity was a little less than that of dimercaprol.

D. α : ω -Dithiols. 1:4-dithiothreitol (0:2) behaved quite differently from its 1:2-isomer, 0:13. It was about 30 per cent. more toxic, and caused violent convulsions and death within 5 or 10 minutes of intramuscular injection. Post-mortem examination of mice killed by the drug and of survivors killed 28 days later, showed nothing macroscopically abnormal. When injected in doses by themselves innocuous, 0:2 greatly accelerated death after oxophenarsine and increased the mortality from sublethal doses. Death in animals so treated was preceded by convulsions, and at post-mortem examination gross pulmonary congestion

and hæmorrhages were observed, with a highly significant increase in lung weight (11.0 ± 0.53 mg/g. compared with 7.9 ± 0.20 mg./g. in mice poisoned with the same dose of oxophenarsine alone). In the occurrence of convulsions, the rapidity of death and the increase in lung weight, poisoning with oxophenarsine and 1:4-dithiothreitol resembles poisoning with the oxophenarsine-dimercaprol compound, and it seems possible that a similar mechanism may underlie both phenomena. 1:4-dithioerythritol (0:22) was considerably less toxic than its stereo-isomer, and caused death less rapidly: but it also accelerated death in oxophenarsine poisoning, though less dramatically. 1:6-dithiodulcitol (0:1) had a particularly low acute toxicity, of about 3.4 mM/kg. But mice receiving doses over about 2 mM/kg. developed a remarkable disease at some time between 3 and 20 days after injection. The hind legs became paralysed and the posterior part of the body slowly wasted until the body weight fell sometimes by as much as 50 per cent. Diarrhoea was common, and a crust of dirt and excreta was usually present at the base of the tail. There was often dermatitis of the tail, and a number of mice developed periorbital abscesses. Death occurred at any time up to at least 28 days after injection and 14 days after the onset of paralysis, but a proportion of mice receiving doses of 2.5 mM/kg. or less recovered and appeared normal 28 days after injection. The latter were killed and were found to have paler and firmer lungs than normal, but no other gross abnormality. Two paralysed mice, killed at the same time, showed gross wasting and contractures, generalised loss of size and weight of the viscera proportional to the body-weight, and no macroscopic lesions of the central nervous system. This dithiol gave slight protection against oxophenarsine poisoning, but some protected mice subsequently developed the paralytic syndrome. The dithiodulcitol was dissolved in a solution containing sodium borate, but no such syndrome developed in mice treated with sodium borate in amounts corresponding to the amounts used in dissolving the dithiodulcitol and no other mice under experiment at the same time developed the same syndrome. Sodium borate gave no protection against oxophenarsine poisoning. A rabbit which received 2 mM/kg. of dithiodulcitol intraperitoneally had much diarrhoea and died within 24 hours with gross lung œdema and congestion. Another rabbit into which 0.2 mM/kg. was injected showed no ill-effects in the next three weeks.

E. Acetyldithiols. The acetyldithiols were all less toxic, on a molecular basis, than the corresponding free dithiols. Some, particularly triacetyl dimercaprol (0:12), had considerable activity against oxophenarsine. Both toxicity and anti-arsenical activity diminished as the molecular weight increased. The lethal doses of 3:4:5-triacetoxy-1:2-bis-acetylthiopentane (0:18) and hexa-acetyl β -(2:3-dimercaptopropyl) glucoside (0:11) were too large to be dissolved in a harmless quantity of any solvent tried, and 0:11 only increased slightly the survival time of oxophenarsine-poisoned mice without saving any lives, while 0:18 was inactive. Triacetyl dimercaprol (0:3) behaved very similarly to

dimercaprol itself. Diacetyl dimercaprol (0:21) was anomalous. The method of synthesis was directed to producing the di-S-acetyl compound, but by iodine titration it appeared to be a monothiol, and one acetyl group had therefore presumably wandered to the oxygen atom. Its toxicity was low, though large doses produced symptoms like dimercaprol; but unlike either dimercaprol or triacetyl dimercaprol, it saved no lives and considerably accelerated death in mice poisoned with oxophenarsine. Tetra-acetyl 1:4-dithiothreitol (0:4) appeared to be less toxic than the free α : ω -dithiol and, like it, potentiated oxophenarsine poisoning.

DISCUSSION

The main object of the work reported here was to examine the possibility that other dithiols might be more satisfactory therapeutic agents than dimercaprol. In order to do so, the toxicity of various dithiols was estimated and their anti-arsenical activity was compared with that of dimercaprol. The quantitative method of assessing anti-arsenical activity described by Weatherall and Weatherall⁸ was not devised until most of the substances described here had been tested, and only approximate comparisons are afforded by the present data. Danielli *et al.*¹³ found that the toxicity of dithiols was decreased by the introduction of hydrophilic groups into the molecule, and this is borne out by the present findings. Unfortunately, the diminution in toxicity has been accompanied by a loss of anti-arsenical activity, at least when oxophenarsine was used as the arsenical. Which loss preponderated depended on how the hydrophilic groups were introduced. Simple lengthening of the chain of a hydroxythiol by the introduction of -CHOH- groups resulted in more loss of activity than of toxicity. On the other hand, conjugation by means of an ether linkage with, for example, a sugar produced dithiols which were substantially less toxic than dimercaprol, but retained quite good activity against oxophenarsine. The most marked defect of the sugar ethers was their inability to save lives when they were given some time after the arsenical poison. Dimercaprol glucoside was clearly less efficient than dimercaprol in this respect, although the difference was less conspicuous with 6(2':3'-dimercaptpropyl) sorbitol. Dimercaprol glucoside was the least toxic of all the dithiols tested, and had otherwise good anti-arsenical activity, and seemed clearly to deserve further investigation from the point of view of possible therapeutic value.

An alternative method of diminishing the toxicity of dithiols lay in masking the -SH groups by some combination which might be readily broken down in the body. As the acetyl derivatives of several of the dithiols studied were readily available, some were tried, and were found to possess moderately good activity against oxophenarsine. The acetylated dithiols of low molecular weight were more effective than larger molecules. The smaller molecules tend to be more soluble in water and may be expected to hydrolyse more readily to the free dithiols, so this difference was not surprising. It is possible that by slow hydrolysis suitable acetylated dithiols would liberate a moderate sustained concentration of

dithiol, which would be therapeutically more useful than the rather short action of dimercaprol. Experiments in which hexa-acetyl β -(2:3-dimercaptopropyl) glucoside (0:11) was given three hours before a dose of oxophenarsine indicated that no protective concentration of thiol had been liberated. Possibly some other acetyl dithiol would have been more effective.

Separation of the -SH groups of dithiols has been found in the three instances studied here to be undesirable. 1:6-dithiodulcitol was only weakly active against oxophenarsine and produced a peculiar wasting disease by some mechanism which has not been examined. Both 1:4-dithiols potentiated oxophenarsine poisoning, in a manner which resembled the toxic effects of the oxophenarsine-dimercaprol compound. The similarity suggested that a common mechanism might be involved. Peters and Stocken⁴ suggested that the compound of oxophenarsine and dimercaprol possibly penetrated cells and there dissociated with the intracellular liberation of toxic arsenic. The 1:4-dithiols are likely to form a less stable ring with arsenic than the 1:2-compounds (Whittaker¹⁵), and possibly form such compounds *in vivo* which penetrate cells and then dissociate again with fatal results. Some support is lent to Peters and Stocken's concept by the harmlessness of the compound of oxophenarsine and dimercaprol glucoside, which is presumably more soluble in water and consequently less liable to enter cells. As indicated above, no evidence at all could be found of any enhancement of toxicity of oxophenarsine by such combination.

SUMMARY

1. The toxicity in mice has been studied of oxophenarsine, the oxophenarsine-dimercaprol compound, 15 dithiols and 10 acetyl dithiols.

2. The LD₅₀ of oxophenarsine given intramuscularly was about 0.14 mM/kg. Significant differences in mortality were not observed when oxophenarsine was given intraperitoneally.

3. Death occurred more rapidly after large than after small doses of oxophenarsine. The lungs of mice dying of oxophenarsine poisoning were heavier than normal, particularly when the doses were small and the time of survival long.

4. The LD₅₀ of the oxophenarsine-dimercaprol compound given intramuscularly was about 0.06 mM/kg. and given intraperitoneally was about 0.30 mM/kg. Death occurred more rapidly than in oxophenarsine poisoning and the lungs were much heavier than either normally or in oxophenarsine poisoning.

5. The LD₅₀ of dimercaprol glucoside was found to depend on the iodine titre of the solution used. Estimated on this basis, the least toxic samples examined had an LD₅₀ of about 4.5 mM/kg.

6. Dimercaprol glucoside was effective against larger doses of oxophenarsine than was dimercaprol when the thiols were given immediately after oxophenarsine. The glucoside was less effective than dimercaprol when the thiols were given more than about 20 minutes after oxophenarsine.

7. Approximately equimolar mixtures of oxophenarsine and dimer-caprol glucoside were not more toxic than oxophenarsine alone.

8. No other dithiols examined had as good a combination of low toxicity and high activity against oxophenarsine as dimercaprol glucoside. The nearest approach was made by other dimercaprol sugar ethers.

9. Acetylated dithiols of low molecular weight had quite good anti-arsenical activity, but were not more active relative to their toxicity than free dithiols.

10. Acetylated dithiols with a molecular weight greater than about 300 had very low toxicity and little or no activity against oxophenarsine.

11. Three substances (diacetyl dimercaprol, 1:4-dithioerythritol and particularly 1:4-dithiothreitol) accelerated death and increased the mortality in mice poisoned with oxophenarsine.

The dimercaprol used in these experiments was a sample of water-purified BAL kindly presented by Professor R. A. Peters. Some of the dimercaprol glucoside was prepared by Dr. L. N. Owen, some was provided by the Ministry of Supply, and one sample was provided by Boots Pure Drug Co., Ltd. The other dithiols and all the acetylated dithiols were prepared by Dr. L. N. Owen and his associates. Oxophenarsine (mapharside, not diluted with sucrose as in commercial preparations) was generously presented by Dr. J. S. White, of Parke Davis and Co., Ltd. The oxophenarsine-dimercaprol compound was synthesised and kindly presented by Dr. L. A. Stocken. I am most grateful for all these gifts. I am much indebted also to Dr. L. N. Owen for numerous helpful discussions, to Mr. Leslie Angus, Miss Jean Tulloch and Miss Irene Munro for technical assistance, to Mrs. J. A. C. Weatherall for performing certain of the toxicity tests, and particularly to Professor J. H. Gaddum for his advice and criticism. The work was initiated during the tenure of a personal grant from the Medical Research Council and was supported by a grant for expenses from the Council.

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THE PARTITION CHROMATOGRAPHY OF ALKALOIDS

PART II.—THE ALKALOIDS OF AUSTRALIAN *DATURA FEROX* AND OF INDIAN HENBANE

BY W. C. EVANS AND M. W. PARTRIDGE

From the University, Nottingham

Received June 17, 1949

IN Part I¹ attention was directed to the possibility that *Datura ferox* may yield different alkaloids when grown in different localities. Australian-grown *D. ferox* was found by Barnard and Finnemore² to contain hyoscyamine as the major alkaloid and material grown in the Crimea afforded, according to Libizov³, hyoscyamine and hyoscyne. We found that plants grown in this country produced hyoscyne as the chief alkaloidal constituent and meteloidine as a relatively minor alkaloid. In view of the well authenticated cases of variation in the nature of the alkaloids within certain species of the Solanaceæ, viz., *Duboisia species* and *Datura metel*⁴ it appeared of interest to accumulate more evidence on this point for *D. ferox*. Moreover, it would appear from the results reported in Part I and to be described in this communication that *D. ferox* may provide a convenient commercial source of hyoscyne.

Samples of Indian henbane are from time to time offered for sale in this country and presumably are used for the manufacture of galenical preparations. As far as we are aware, no information is available either on the plant source of these materials⁵ or on the nature of the alkaloids contained in them. Accordingly a comparison has been made of the alkaloids of Indian henbane with those of authentic *Hyoscyamus niger*.

EXPERIMENTAL

The Australian *D. ferox* available consisted of about 120 g. of whole plant grown at Canberra and seeds from plants grown at Shepparton, Victoria. The Indian henbane was a commercial sample purchased in this country. The botanical source of this material is doubtful but we are informed by Mr. J. L. Forsdike that it differs from *H. niger*.

Quantities of 100 g. of both the whole plant and the seeds of *D. ferox* were extracted by a modification of the Pharmacopœial assay process⁶. An ethereal solution of the total alkaloids was in each case chromatographically fractionated first with ether and then with chloroform on a column of 20 g. of kieselguhr on which was distributed 10 ml. of M/2 phosphate buffer of pH 7.3. Details of this technique and the method of collecting fractions of the eluate are described in Part I. The alkaloids in the fractions of eluting solvent were identified as their picrates.

For comparison of the alkaloids of Indian henbane with those of *H. niger*, 500 g. of the Indian drug and 100 g. of *H. niger* were extracted and chromatographically fractionated by the same procedure, the M/2 phosphate buffer having pH 6.8 in this instance. Because of the low proportion of alkaloids in the Indian henbane, a total of 10 kg. was extracted in batches of 2 kg. for the identification of the alkaloids after

chromatographic fractionation. The titration liquors corresponding to a given peak were combined; the aqueous layer, containing the alkaloidal sulphate was separated from the organic solvent and indicator was removed from it by shaking with chloroform; after concentrating under reduced pressure, the solution was available for the preparation of solid derivatives by double decomposition with the appropriate reagent.

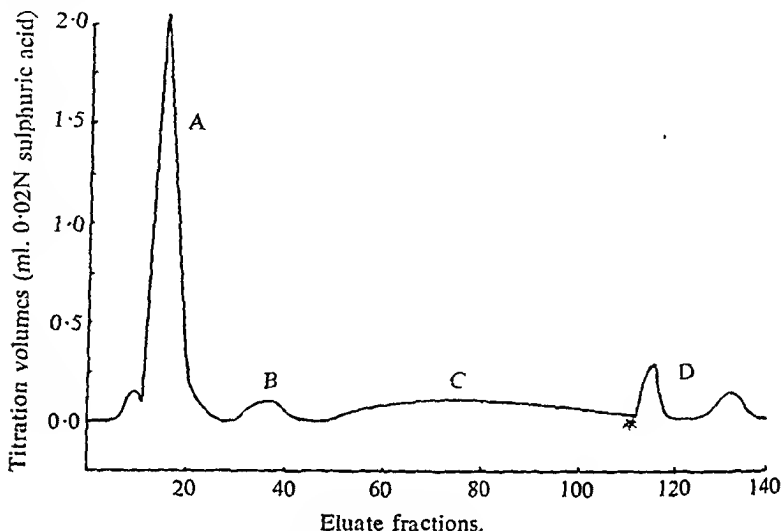


FIG. 1.—Separation of alkaloids from 100g. of Australian *Datura ferox* (whole plant)

Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 7.3.

* Chloroform B.P. used as eluant.

Figure 1 shows the separation of the alkaloids of the whole plant of *D. ferox*. The eluate fractions represented by the large peak A were shown to contain hyoscyne by the preparation of the picrate, m.pt. 187°C ., not depressed on admixture with authentic hyoscyne picrate. The proportion of hyoscyne found by titration was 0.06 per cent, and by isolation of the picrate, 0.05 per cent. From the eluate fractions corresponding to peak B, 5 mg. of a picrate, m.pt. 230°C ., was isolated. Fractions 52 to 112 of the eluate (peak C) afforded meteloidine picrate, m.pt. 174° to 175°C ., undepressed on admixture with authentic material. The melting-point of this picrate was considerably depressed when mixed with hyos-

TABLE I

Alkaloid	<i>H niser</i>	Indian henbane
Total alkaloids, calculated as hyoscyamine	per cent 0.073	per cent 0.031
Hyoscyne	0.028	0.016
Hyoscyamine	0.041	0.010
Tropine	0.0025	0.0015

cyamine picrate or with atropine picrate. The weight of meteloidine picrate isolated was 3 mg.: no reasonably accurate estimation of the proportion of this alkaloid was possible by a summation of the very small,

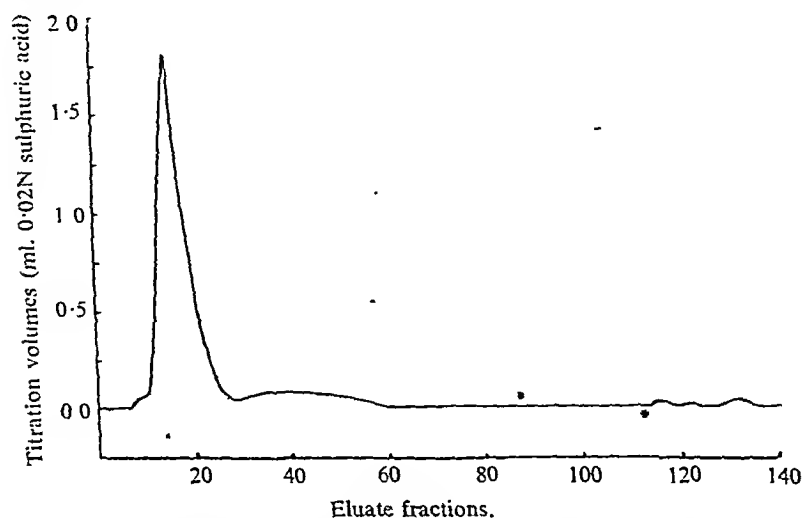


FIG. 2.—Separation of alkaloids from 100g. of Australian *Datura ferox* seeds.

Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 7.3.

* Chloroform B.P. used as eluant.

individual eluate-fraction titres. The partition chromatogram of *D. ferox* seeds is represented in Figure 2. The only significant peak was shown to refer to hyoscine, 0.093 per cent., which afforded hyoscine

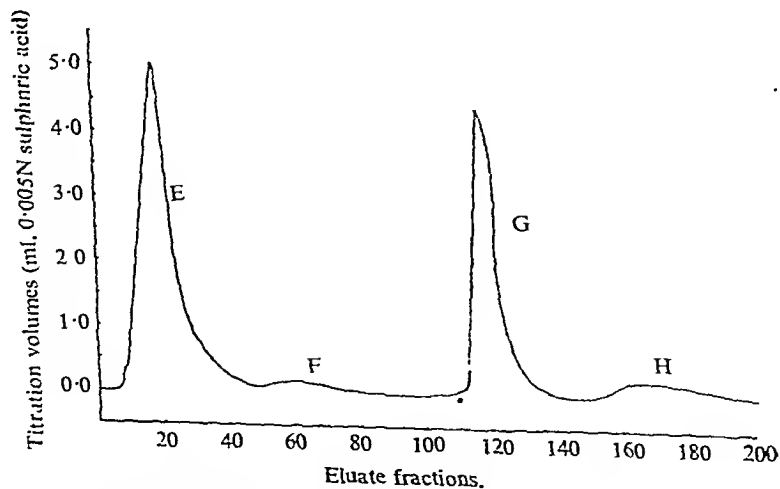


FIG. 3.—Separation of alkaloids from 100g. of Indian henbane.
Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 6.8.

* Chloroform B.P. used as eluant.

chromatographic fractionation. The titration liquors corresponding to a given peak were combined; the aqueous layer, containing the alkaloidal sulphate was separated from the organic solvent and indicator was removed from it by shaking with chloroform; after concentrating under reduced pressure, the solution was available for the preparation of solid derivatives by double decomposition with the appropriate reagent.

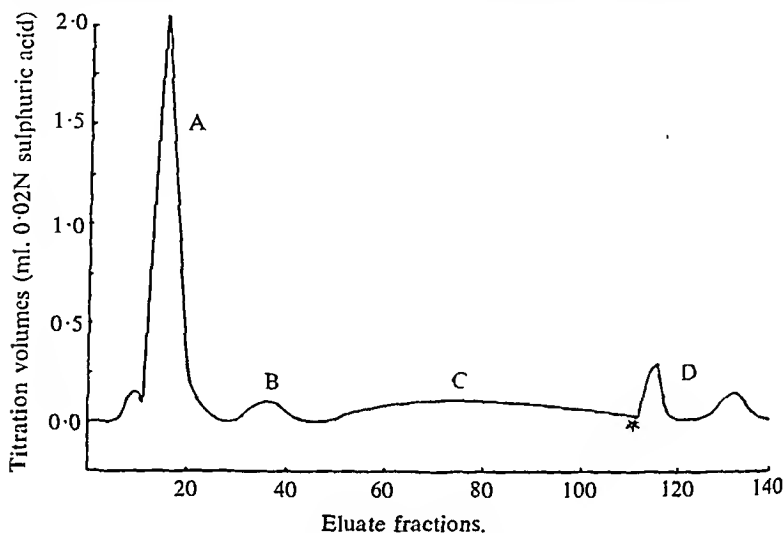


FIG. 1.—Separation of alkaloids from 100g. of Australian *Datura ferox* (whole plant)

Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 7.3.

* Chloroform B.P. used as eluant.

Figure 1 shows the separation of the alkaloids of the whole plant of *D. ferox*. The eluate fractions represented by the large peak A were shown to contain hyoscyne by the preparation of the picrate, m.pt. 187°C., not depressed on admixture with authentic hyoscyne picrate. The proportion of hyoscyne found by titration was 0.06 per cent. and by isolation of the picrate, 0.05 per cent. From the eluate fractions corresponding to peak B, 5 mg. of a picrate, m.pt. 230°C., was isolated. Fractions 52 to 112 of the eluate (peak C) afforded meteloidine picrate, m.pt. 174° to 175°C., undepressed on admixture with authentic material. The melting-point of this picrate was considerably depressed when mixed with hyos-

TABLE I

Alkaloid	<i>H. niger</i>	Indian henbane
Total alkaloids, calculated as hyoscyamine	per cent 0.073	per cent 0.031
Hyoscyne	0.028	0.016
Hyoscyamine	0.041	0.010
Tropine	0.0025	0.0015

THE PARTITION CHROMATOGRAPHY OF ALKALOIDS—PART II

cyamine picrate or with atropine picrate. The weight of meteloidine picrate isolated was 3 mg.; no reasonably accurate estimation of the proportion of this alkaloid was possible by a summation of the very small,

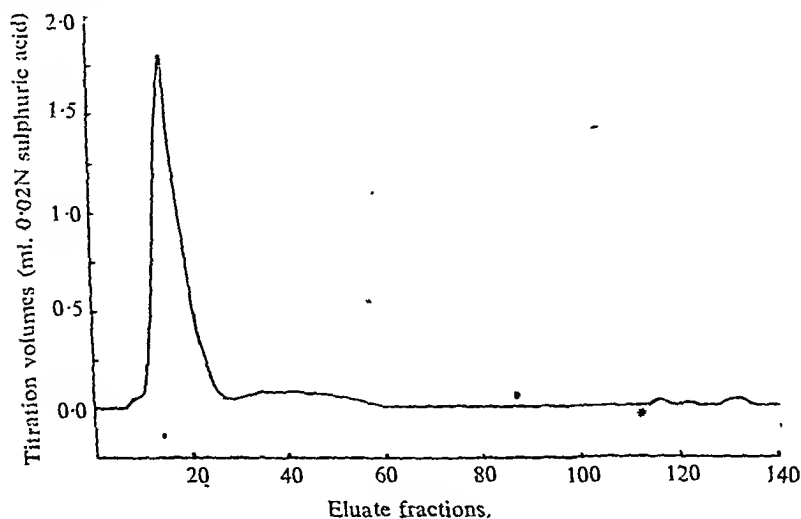


FIG. 2.—Separation of alkaloids from 100g. of Australian *Datura ferox* seeds.

Column, 20g. Kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 7.3.

* Chloroform B.P. used as eluant.

individual eluate-fraction titres. The partition chromatogram of *D. ferox* seeds is represented in Figure 2. The only significant peak was shown to refer to hyoscyine, 0.093 per cent., which afforded hyoscyine

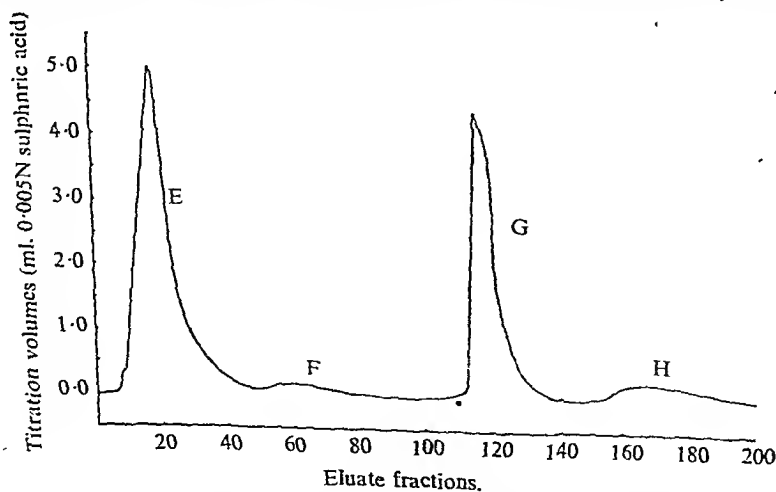


FIG. 3.—Separation of alkaloids from 100g. of Indian henbane.
Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 6.8.

* Chloroform B.P. used as eluant.

picrate, m.pt. 187°C ., undepressed by authentic hyoscyne picrate. The proportion of total alkaloids, calculated as hyoscyne, was 0.095 per cent.

For Indian henbane and *H. niger*, the partition chromatograms were as shown in Figures 3 and 4. Quantitative data obtained from the respective 500 g. and 100 g. quantities of these two drugs are summarised in Table I. The shape of the chromatogram curve from the larger-scale extraction of Indian henbane was similar to Figure 3.

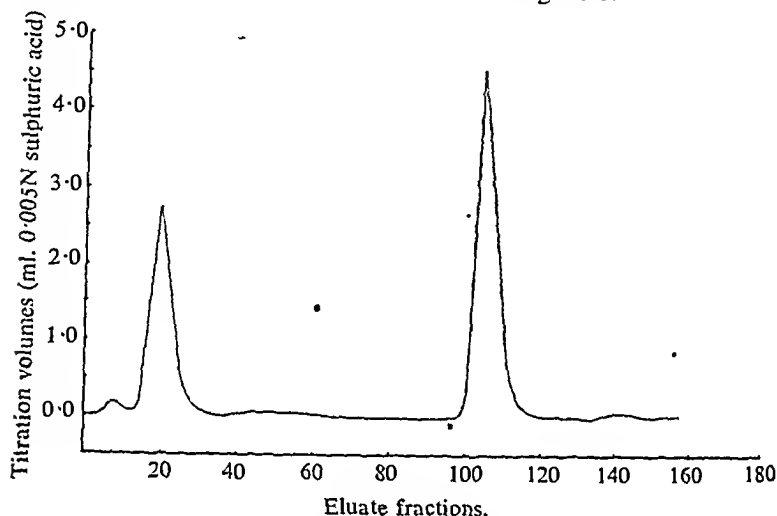


FIG. 4.—Separation of alkaloids from 100g. of *Hyoscyamus niger*.
Column, 20g. kieselguhr with 10 ml. 0.5 M phosphate buffer, pH 6.8.
* Chloroform B.P. used as eluant.

Peak E was demonstrated to correspond to hyoscyne by the preparation of the picrate, m.pt. and mixed m.pt. 186°C . and the aurichloride, m.pt. and mixed m.pt. 204°C ., with decomposition. From the fractions represented by peak F, a picrate, m.pt. 230°C ., was isolated; this material was identical with the picrate of the same melting-point isolated from *D. ferox*. The alkaloid corresponding to peak G was found to be hyoscyamine, since it afforded an aurichloride, m.pt. 163°C ., not depressed on admixture with authentic hyoscyamine aurichloride, and also a picrate, m.pt. 164° to 165°C ., not depressed by mixing with authentic material. For the identification of the alkaloid corresponding to peak H, the auribromide and Reineckate were found to be satisfactory. The auribromide crystallised from water or dilute hydrobromic acid as dark brown needles, m.pt. 196°C ., with decomposition. Found: C, 14.9; H, 2.4; N, 2.4 per cent. $\text{C}_5\text{H}_{15}\text{ON}, \text{HAuBr}$, requires C, 14.6; H, 2.4; N, 2.1 per cent. The melting-point of this substance was undepressed on admixture with the auribromide prepared from tropine made by the hydrolysis of hyoscyamine according to Ladenburg⁷. The Reineckate, m.pt. 239° to 240°C ., with decomposition, did not depress the melting-point of authentic tropine Reineckate¹. Found: N, 20.3 per cent. $\text{C}_5\text{H}_{15}\text{ON}, \text{H}[\text{Cr}(\text{SCN})_4(\text{NH}_2)]$. H_2O requires N, 20.5 per cent.

DISCUSSION OF RESULTS

Contrary to previous findings, the experiments described in this communication and in Part I¹ show that samples of *D. ferox* grown in Australia and in this country contain hyoscyne as the principal alkaloidal constituent. Its proportion is very variable since material grown in Nottingham contained 0.4 per cent, whereas the Australian material contained only 0.06 per cent. This plant is common in Australia and in view of the low proportion of other alkaloidal constituents, may, if the constancy of alkaloidal type is confirmed for other Australian samples, provide a convenient commercial source of hyoscyne. Since the partition chromatogram indicated the complete absence of meteloidine from the seeds, it would appear that this alkaloid is not a product of metabolism of the reproductive organs of the plant. It is hoped that further information on the nature of the alkaloid which afforded a picrate, m.pt. 230° C., and on the alkaloids of *D. ferox* which are eluted with chloroform (peak D) will be presented in a further communication.

The major alkaloids of Indian henbane are hyoscyne and hyoscyamine with tropine as a minor alkaloid, but the total alkaloidal content is very low. Notwithstanding anatomical differences, the shape of the partition chromatogram curve, which is a function of the R_f values characteristic of the alkaloids, demonstrates that the only significant difference between Indian henbane and *H. niger* is in the relative proportions of the alkaloids. The small final peak in the curve for *H. niger* is likely to correspond to tropine since a comparison of the curves for *H. niger* and Indian henbane shows that the R_f value must be very close to that of the tropine independently identified in the Indian henbane. This sample of Indian henbane bore no resemblance in its constituents to *H. reticulatus*, which according to Konovalova and Magidson³, contains hyoscyamine and 1:4-bisdimethylaminobutane. The characterisation of the small quantity of tropine presented some difficulty. The picrate and aurichloride were too soluble to afford convenient derivatives and the auribromide decomposed slightly on repeated recrystallisation. We now find that the melting-point of the Reineckate varies appreciably with the rate of heating; in Part I the value recorded was 251° to 252°C., with decomposition, after sintering at 246° to 248°C., whereas the best reproducible value is probably 245° to 246°C., with decomposition.

We are greatly indebted to Dr. C. Barnard of the Council for Scientific and Industrial Research, Australia for providing the samples of *D. ferox* and to Mr. J. L. Forsdike for procuring the sample of Indian henbane.

SUMMARY

1. Australian-grown *Datura ferox* has been found to contain as principal alkaloids, hyoscyne and meteloidine.
2. The chief alkaloidal constituents of Indian henbane are hyoscyne, hyoscyamine and tropine; in this respect, Indian henbane differs from *Hyoscyamus niger* only in the absolute and relative proportions of the

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THE ASSAY OF MERCURIC SALICYLATE

By M. DOMBROW

From the Chelsea School of Pharmacy

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THE iodometric method of assaying mercuric salicylate of the British Pharmaceutical Codex, 1934, was replaced in the Fourth Supplement by a thiocyanate titration of the mercury. The organic matter is destroyed by oxidation with potassium permanganate, first in alkaline then in acid solution and excess of oxidising agent is removed by adding ferrous sulphate solution.

Several other standard procedures for the determination of mercury in organic compounds or in the presence of organic matter follow the same principle, but oxidation is usually effected by heating with a mixture of nitric and sulphuric acids.^{1, 2, 3} While it is accepted that salicylates and other phenols can be oxidised by permanganate, there is no specific information in the literature on the oxidation of mercuric salicylate. The composition of this salt has not yet been established and though it is described as a salt of salicylic acid in which the mercury replaces both the phenolic and carboxylic hydrogen atoms⁴, the composition varies with the method of preparation and the B.P.C. allows material containing between 54 and 59.6 per cent. of mercury.

It was found in a preliminary experiment that a carefully homogenised sample assayed differently by the B.P.C. and United States Pharmacopœia XI methods. The latter employs a nitric-sulphuric acid oxidation followed by a thiocyanate titration of the mercury. The results differed by 1.27, representing a divergence of over 2 per cent. of the mercury. The mercury content of the sample was established gravimetrically by decomposing the sample with boiling hydrochloric acid and precipitating as sulphide. The precipitate was filtered, washed and dried to constant weight. A carbon disulphide extraction showed that no sulphur had been precipitated. The results were in close agreement with the U.S.P. figures: B.P.C. method, 56.30 per cent.; U.S.P. XI method 57.57 per cent.; sulphide method 57.55 per cent. (mean of 3 determinations).

BRITISH PHARMACEUTICAL CODEX METHOD

Dissolve 0.3 g. of sample in 10 ml. of 0.1N sodium carbonate. Add 1.5 g. of finely powdered potassium permanganate in small portions and mix well. After 5 minutes add carefully 5 ml. of sulphuric acid and after a further 5 minutes, 40 ml. of water and then acid solution of ferrous sulphate in small quantities, shaking after each addition until the precipitate is dissolved. Add 5 ml. of nitric acid and titrate with 0.1N ammonium thiocyanate, using ferric ammonium sulphate as indicator.

Acid Solution of Ferrous Sulphate. Freshly prepared by dissolving 7 g. of ferrous sulphate in 90 ml. of water, freshly boiled and cooled, and adding sulphuric acid to 100 ml.

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THE ASSAY OF MERCURIC SALICYLATE

By M. DOMBROW

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THE iodometric method of assaying mercuric salicylate of the British Pharmaceutical Codex, 1934, was replaced in the Fourth Supplement by a thiocyanate titration of the mercury. The organic matter is destroyed by oxidation with potassium permanganate, first in alkaline then in acid solution and excess of oxidising agent is removed by adding ferrous sulphate solution.

Several other standard procedures for the determination of mercury in organic compounds or in the presence of organic matter follow the same principle, but oxidation is usually effected by heating with a mixture of nitric and sulphuric acids.^{1, 2, 3} While it is accepted that salicylates and other phenols can be oxidised by permanganate, there is no specific information in the literature on the oxidation of mercuric salicylate. The composition of this salt has not yet been established and though it is described as a salt of salicylic acid in which the mercury replaces both the phenolic and carboxylic hydrogen atoms⁴, the composition varies with the method of preparation and the B.P.C. allows material containing between 54 and 59.6 per cent. of mercury.

It was found in a preliminary experiment that a carefully homogenised sample assayed differently by the B.P.C. and United States Pharmacopœia XI methods. The latter employs a nitric-sulphuric acid oxidation followed by a thiocyanate titration of the mercury. The results differed by 1.27, representing a divergence of over 2 per cent. of the mercury. The mercury content of the sample was established gravimetrically by decomposing the sample with boiling hydrochloric acid and precipitating as sulphide. The precipitate was filtered, washed and dried to constant weight. A carbon disulphide extraction showed that no sulphur had been precipitated. The results were in close agreement with the U.S.P. figures: B.P.C. method, 56.30 per cent.; U.S.P. XI method 57.57 per cent.; sulphide method 57.55 per cent. (mean of 3 determinations).

BRITISH PHARMACEUTICAL CODEX METHOD

Dissolve 0.3 g. of sample in 10 ml. of 0.1N sodium carbonate. Add 1.5 g. of finely powdered potassium permanganate in small portions and mix well. After 5 minutes add carefully 5 ml. of sulphuric acid and after a further 5 minutes, 40 ml. of water and then acid solution of ferrous sulphate in small quantities, shaking after each addition until the precipitate is dissolved. Add 5 ml. of nitric acid and titrate with 0.1N ammonium thiocyanate, using ferric ammonium sulphate as indicator.

Acid Solution of Ferrous Sulphate. Freshly prepared by dissolving 7 g. of ferrous sulphate in 90 ml. of water, freshly boiled and cooled, and adding sulphuric acid to 100 ml.

THIOCYANATE TITRATION CONDITIONS

The acidity and amount of indicator added in the titration conform to established usage^{5,6,7,8}, but about 70 ml. of ferrous sulphate solution is required to dissolve the manganese dioxide precipitate, introducing ferric ion equivalent to 8.5 g. of ferric ammonium sulphate or 85 ml. of indicator solution. Variation of the indicator concentration has been found of little significance over a small range⁸, but the total ferric concentration here is far above any hitherto tested. Furthermore, the sensitivity of the end-point is reduced in the strongly-coloured solution.

The quantitative effect was determined by titrating a standard solution of mercuric nitrate with thiocyanate at the same final mercury concentration and acidity as in the B.P.C. method using (a) 90 ml. of indicator, (b) 5 ml. of indicator and 85 ml. of water; results: (a) 17.50 ml.; (b) 17.65 ml.

The end-point, taken as the first definite brownish colour, was premature and represented a loss of 1 per cent. on the low burette reading. This accounts for part, at least, of the deficiency shown.

In all subsequent work, the ferrous sulphate solution was replaced by 3 per cent. hydrogen peroxide, added in small portions until the solution was clear; excess of peroxide was removed with 10 per cent. permanganate solution, added to the first permanent pink and decolorised with a small crystal of ferrous sulphate. The suitability of this modification was verified by titrating standard mercuric nitrate with thiocyanate (a) alone (b) in the presence of a solution prepared by carrying out a blank determination on salicylic acid, using the modified method. Identical results were obtained.

QUANTITY OF PERMANGANATE

The bulk of the oxidation occurs in alkaline solution, a heavy brown precipitate of manganese dioxide being thrown down immediately on adding permanganate. The solution becomes purple when all the permanganate has been introduced and the colour and precipitate remain until the reducing agent is added. The excess of permanganate is small since the purple colour fails to develop on increasing the weight of sample by less than 10 per cent.

The relation between sample weight/permanganate ratio and completeness of oxidation was studied in a series of determinations in which the weight of permanganate was fixed at 1.5 g. and the weight of sample varied from 0.2 to 0.45 g. The results were calculated in terms of burette reading per g. of sample.

Reduction of the sample weight below 0.3 g. had no effect on the recovery, whereas from 0.32 g. upwards, low and erratic results were obtained. An adequate safety margin was established by using 2 g. of permanganate, with which recoveries were fully maintained up to 0.4 g. of sample.

ASSAY OF MERCURIC SALICYLATE

TIME AND TEMPERATURE OF OXIDATION

Increasing the alkaline oxidation time to 25 minutes had no significant effect (Table I). The importance of the acid oxidation stage is shown by the added recovery on increasing the severity of the conditions. Whilst a longer period than the 5 minutes oxidation of the B.P.C. method gave by itself no advantage, due probably to the rapid fall of temperature in the initial few minutes, a higher recovery was obtained on heating the solution for 10 minutes (Table I).

TABLE I

Conditions of oxidation						Mercury per cent.
Alkaline soln.			Acid soln.		Temp. effect	
25 minutes	5 minutes	1	Not heated	57.32
5 "	5 "	5	"	56.94
5 "	5 "	10	"	57.44
5 "	5 "	5	Heated	57.43
5 "	5 "	10	"	57.45
5 "	5 "	20	"	57.59
5 "	5 "	20	"	57.59

LOSSES DURING ACIDIFICATION

Acidification with sulphuric acid produces violent effervescence; the liquid boils and purple fumes are given off. Cooling is rapid in 5 minutes, owing to the relatively large surface area of the small volume of liquid. It was found that 10 ml. of 50 per cent. sulphuric acid gave a strong and more prolonged heat effect and enabled the reaction to be easily controlled, with little spray and no fuming. Recoveries were comparative both with the unheated solutions and on heating for 10 minutes, as suggested in the previous section. Results: sulphuric acid B.P. unheated 57.44 per cent., heated 10 minutes 57.59 per cent.; sulphuric acid (50 per cent.) unheated, 57.43 per cent., heated 10 minutes, 57.57 per cent.

MODIFIED METHOD

Dissolve 0.3 g. of sample, accurately weighed, in 10 ml. of N (approx.) sodium carbonate. Add in small portions 2 g. of finely powdered potassium permanganate and mix well. After 5 minutes, add carefully 10 ml. of sulphuric acid (50 per cent.) and boil the solution gently for 10 minutes. Cool, add 40 ml. of water and then hydrogen peroxide solution (3 per cent.) in small portions until the precipitate is dissolved. Add potassium permanganate solution (10 per cent.) until the solution becomes a faint permanent pink, decolorise with a small crystal of ferrous sulphate, add 5 ml. of nitric acid and titrate with 0.1N ammonium thiocyanate solution using ferric ammonium sulphate solution as indicator.

The modified method was examined on a range of samples of varying mercury content (Table II), and found to agree closely with the U.S.P. XI and sulphide methods.

Table II also shows that the precision of the modified method is higher than that of the U.S.P. XI and B.P.C. methods.

TABLE II

Sample	Mercury per cent.			
	B.P.C. method	Modified method	U.S.P. XI method	Sulphide method
1	54.53 54.62	55.65 55.67	55.72 55.65	55.55
2	55.29 55.19	56.49 56.44	56.49 56.43	56.45
3	55.98 55.93	57.08 57.11	57.15 57.08	56.99
4	56.29 56.25 56.36	57.57 57.59 57.56	57.68 57.59 57.44	57.57 57.49 57.59
5	57.08 57.04	58.38 58.41	58.44 58.34	58.32
6	58.18 58.29	59.48 59.53	59.51 59.51	59.48

SUMMARY

1. The B.P.C. assay of mercuric salicylate has been shown to give low results, compared with the U.S.P. XI method and a gravimetric sulphide method.

2. A study of the conditions of the B.P.C. method has been made and sources of error revealed.

3. A modification of the B.P.C. method has been proposed which gives results in agreement with the sulphide and U.S.P. XI methods.

I am indebted to Mr. C. Morton, B.Sc., Ph.C., Head of the Chelsea School of Pharmacy, for his helpful criticisms.

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THE PROPERTIES AND REACTIONS OF DECAMETHONIUM IODIDE AND HEXAMETHONIUM BROMIDE

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THE work of Paton and Zaimis¹ on decamethonium iodide (α : ω -hexamethyldiaminodecane diiodide, known as C.10) showed that it was a very potent substance, and that it was capable of replacing *d*-tubocurarine chloride in medicine. An antidote exists for decamethonium iodide in hexamethonium bromide (α : ω -hexamethyldiaminohexane dibromide, known as C.6), and owing to its pharmacological properties this substance has been suggested for use in hypertension and vascular diseases, thereby replacing tetraethylammonium iodide. Hexamethonium bromide is 10 to 20 times as active as the tetraethyl compound. In view, therefore, of the possible importance of the new drugs, it was considered desirable that an examination should be made of their physical and chemical properties.

DECAMETHONIUM IODIDE

Decamethonium iodide is a colourless, odourless, crystalline powder, which when dried for 4 hours at 70°C./20 mm. pressure and then placed in a melting-point apparatus at room temperature, heated rapidly to 235°C. and then at 2°/minute, had m.pt. 245° to 246°C. (corr.). If, however, it was put in the bath at 230°C., it melted at once with decomposition. For analysis it was dried at 70°C. *in vacuo*. Found: C, 38.25; H, 7.45; N, 5.14; I, 47.8 per cent.; $C_{16}H_{36}N_2I_2$ requires C, 37.5; H, 7.4; N, 5.47; I, 49.6 per cent.

It is soluble in water (21°C.) 1 g. in 10 ml. and at 100°C., 5 g. in 1 ml. A 1 per cent. solution is clear and colourless and has pH 6.6; a 10 per cent. solution has pH 6.4. The sterilised ampouled 0.1 per cent. solution in physiological saline solution has pH 6.06. In ethyl alcohol at 20°C., its solubility is less than 1 g. in 100 ml., and at 78.5°C. 1 g. in 3 ml., whereas in methyl alcohol (20°C.) it is 1 g. in 40 ml. and at 64.1°C. 1 g. in 1 ml. In boiling acetone, benzene, chloroform and ether the solubility is less than 1 g. in 500 ml. At 100°C. the material lost 0.26 per cent. and turned a yellow colour. On ignition no ash remained.

Reactions.

Effect of heat.

The solid melts, appears to boil and finally chars. No iodine evolved.

Concentrated sulphuric acid. Cold or hot.

Effervescence. Red brown colour changing to dull violet. Violet colour extracted by carbon tetrachloride to give violet extract.

Concentrated nitric acid.

Cold. Effervescence. Brown colour. Insoluble brown particles. On shaking with carbon tetrachloride violet solution obtained.

Hot. As cold, but solution became colourless and iodine sublimed.

Sodium hydroxide solution 20 per cent. Immediate white precipitate, which redissolved on heating and reappeared on cooling again.

Aqueous sodium nitrite solution. Cold. Immediate brown colour. Violet extract in carbon tetrachloride.

0.1 N silver nitrate. Pale yellow precipitate not soluble in strong solution of ammonia.

Folin-Ciocalteu phenol reagent. Little or no visible change.

Acid potassium iodate solution. Brown precipitate. Completely soluble in carbon tetrachloride to violet solution. Probably iodine.

Reinecke salt solution, 4 per cent. Pink precipitate produced at once.

Saturated aqueous picric acid solution. Bright yellow precipitate, m.pt. 146° to 148°C. Not changed by recrystallisation from methyl alcohol.

Saturated aqueous picronic acid solution. No immediate precipitate. Slow crystallisation on standing. Dull yellow crystals m.pt. 238 to 239°C.

Aqueous gold chloride solution, 10 per cent. Immediate brown, precipitate, m.pt. 170°C. Recrystallised from aqueous alcohol, m.pt. 174°C.

Aqueous platinum chloride solution, 10 per cent. Chocolate-brown precipitate m.pt. 300°C.

Mayer's reagent. Pale yellow precipitate.

Halogen determination 98.74 per cent. purity.
(Volhard method).

Stability of solution. Solutions at concentrations of 1 in 250, 1 in 500 and 1 in 1000 in physiological saline solution were heated at 95° to 100°C. for 30 minutes and kept in sealed tubes for 8 weeks, some in a cool dark place and others exposed to daylight at room temperature. All the solutions remained clear and colourless.

Assay for non-quaternary material. 0.2 g., accurately weighed, was added to a separating funnel containing water (200 ml.), followed by saturated sodium bicarbonate solution (5 ml.) and extracted with chloroform (3 × 20 ml.). The combined chloroform extracts were

DECAMETHONIUM IODIDE AND HEXAMETHONIUM BROMIDE

washed with water (10 ml.), filtered through a plug of cotton wool into a tared beaker, and evaporated and dried at 100°C. for 1 hour. Residue on original material, 0.15 per cent.

HEXAMETHONIUM BROMIDE

Hexamethonium bromide is a colourless, odourless, crystalline powder which, when dried for 2 hours at 95°C./20 mm. pressure and placed in a melting-point apparatus at 150°C. or below, heated rapidly to 260°C. and then at 2°/minute, had m.p.t. 272°C. (decomp., corr.). If it was put in the bath above 230°C. it sintered slightly and then had m.p.t. 273°C. (decomp.) This salt has a tendency to take up moisture, as shown by the sintering which is observed when its m.p.t. is taken some weeks later. For analysis it was dried at 100°C. *in vacuo*. Found: C, 39.9; H, 8.18; N, 7.95; Br, 43.8 per cent.; $C_{12}H_{30}N_2Br_2$ requires C, 39.77; H, 8.28; N, 7.73; Br, 44.21 per cent.

It is soluble in water (21°C.) 1 g. in 1 ml. and at 100°C. 5 g. in 1 ml. A 1 per cent. solution is clear and colourless and has pH 6.56; a 10 per cent. solution has pH 6.0. The sterilised ampouled 1 per cent. solution in physiological saline solution has pH 6.0. In ethyl alcohol at 20°C. its solubility is 1 g. in 30 ml. and at 78.5°C. 1 g. in 3 ml., whereas in methyl alcohol (20°C.) it is 1 g. in 5 ml. and at 64.1°C. 1 g. in 1 ml. It is insoluble in acetone, benzene, chloroform and ether, both hot and cold. At 100°C. the material lost 0.64 per cent. On ignition the ash content was 0.02 per cent.

Reactions.

Effect of heat. Solid melts and blackens.

Concentrated sulphuric acid.

Cold. Light yellow colouration.
Hot. Very slight colour.

Concentrated nitric acid.

Cold or Hot. Deep yellow. Bromine liberated.

Sodium hydroxide solution. White precipitate which dissolved on heating.

Aqueous sodium nitrite solution, 20 per cent. Cold. No apparent reaction, very slight yellow colour.

0.1 N. silver nitrate. Yellow precipitate soluble in concentrated ammonium hydroxide.

Folin-Ciocalteu phenol reagent. Colour changes to pale yellow after boiling, but on standing in the cold becomes turquoise.

Acid potassium iodate solution. No reaction—cold or hot.

Reinecke salt solution, 4 per cent. Pink precipitate formed immediately. The solution on standing did not change.

Saturated aqueous picric acid solution.	Bright yellow precipitate, m.pt. 232° to 234°C. Recrystallisation from methyl alcohol did not change m.pt.
Saturated aqueous picronic acid solution.	No immediate precipitate. Crystallised slowly on standing to give greenish-yellow crystals m.p.t 243°C. (decomp.).
Aqueous gold chloride solution, 10 per cent.	Immediate orange - brown precipitate m.pt. 259°C. (decomp). Recrystallised from 50 per cent. aqueous ethyl alcohol, m.pt. 260°C. (decomp).
Aqueous platinum chloride solution, 10 per cent.	Immediate buff-coloured precipitate m.pt. 300°C.
Mayer's reagent.	Yellow precipitate.
Halogen determination (Volhard method).	99.15 per cent. purity.

Stability of solution. Solutions at concentrations of 1 in 250, 1 in 500 and 1 in 1000 in physiological saline were heated at 95° to 100°C. for 30 minutes, and kept in sealed tubes for 8 weeks, some in a cool dark place and others exposed to daylight at room temperature. All the solutions remained clear and colourless.

Assay for non-quaternary material. 0.2 g., accurately weighed, was added to a separating funnel containing water (200 ml.), followed by saturated sodium bicarbonate solution (5 ml.) and extracted with chloroform (3 × 20 ml.). The combined chloroform extracts were washed with water (10 ml.), filtered through a plug of cotton wool into a tared beaker, and evaporated and dried at 100°C. for 1 hour. Residue on original material, 0.15 per cent.

We are indebted to Mr. F. Ridgway for carrying out some of the determinations.

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THE EVALUATION OF THE BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL AND SOME OF ITS MONOALKYL ETHERS AGAINST *BACTERIUM COLI*

PART VII

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IN the previous paper¹, disinfection data from the reaction between *Bact. coli* and the monoalkyl ethers were analysed statistically. Mean probit-log. time regressions were calculated for each substance and confidence limits for the estimation determined. This present communication is devoted to the analysis of the disinfectant data of experiments conducted at 30°C.; the statistical methods used follow exactly those in Part VI of this series of papers¹.

DISINFECTION STUDIES OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 30°C.

Probit-log. time regressions at 30°C.

EXPERIMENTAL

Concentrations of ethylene glycol and the ethers were prepared and their disinfectant activity tested against *Bact. coli* at 30°C. by means of the standardised technique². In most instances four tests were carried out on each concentration.

RESULTS AND CALCULATIONS

Probit-log. time regressions were calculated for each test. Summaries of the terms necessary to calculate the mean slopes and the error mean square at each concentration are presented in Tables IA to VID.

TABLE IA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL AT 30°C.

Observation	Concentrations of ethylene glycol			
	62.5 per cent.	65.0 per cent.	67.5 per cent.	70.0 per cent.
$S[(x-\bar{x})(y-\bar{y})]$	1 591879	5 869394	2 657648	4 573893
$S(x-\bar{x})^2$	1 003779	4 035156	2 876413	3 440898
$S(y-\bar{y})^2$	3 229122	9 206112	3 206060	6 590027
N	5	13	13	12
SS for individual regressions	3 199891	8 641415	3 008845	6 298689
b	1 588894	1 454565	0 923945	1 329273
SS pool	2 524539	8 537411	2 488896	6 079953

TABLE 1b

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL AT 30°C.

Concentration	Item	Sum of squares	N	Mean square
62.5 per cent.	Common regression ...	2.524539	1	2.524539
	Variation in regression ...	0.675352	2	0.337676
	Total ...	3.199891	3	
	Residual in y ...	0.029231	5	0.005846
65.0 per cent.	Common regression ...	8.537411	1	8.537411
	Variation in regression ...	0.104004	3	0.034668
	Total ...	8.641415	4	
	Residual in y ...	0.564697	13	0.043438
67.5 per cent.	Common regression ...	2.488896	1	2.488896
	Variation in regression ...	0.519949	5	0.103889
	Total ...	3.008845	6	
	Residual in y ...	0.197215	12	0.016434
70.0 per cent.	Common regression ...	6.079953	1	6.079953
	Variation in regression ...	0.218736	3	0.072912
	Total ...	6.298689	4	
	Residual in y ...	0.291338	12	0.024278

TABLE 1c

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF CONCENTRATIONS OF ETHYLENE GLYCOL AT 30°C.

Concentration	Residual in y		Variation in b		SS pooled b	S[(x-x̄)(y-ȳ)]	S(x-x̄)²
	SS	N	SS	N			
62.5 per cent.	0.029231	5	0.675352	2	2.524539	1.591879	1.003879
65.0 per cent.	0.564697	13	0.104004	3	8.537411	5.869394	4.035156
67.5 per cent.	0.197215	12	0.519949	5	2.488896	2.657648	2.876413
70.0 per cent.	0.291338	12	0.218736	3	6.079953	4.573893	3.440898
Totals ...	1.082481	42	1.518041	13	19.630799	14.692814	11.356346

$$\bar{b} = \frac{14.692814}{11.356346} = 1.293798$$

$$SS \text{ for joint regression} = \frac{(14.692814)^2}{11.356346} = 19.009529$$

TABLE 1d

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL AT 30°C.

Item	N	Sum of squares	Mean square
Grand regression ...	1	19.009529	19.009529
Variation in regression between concentrations ...	3	0.621270	0.207090
Variation in regression within concentrations ...	13	1.518041	0.116772
Residual in y ...	42	1.082481	0.025773

BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL—PART VII

Combined data from the calculations of the probit-log. time regressions for ethylene glycol and its monoalkyl ethers at 30°C.

Table VII presents a summary of the massed statistical data from the calculations of probit-log. time regressions for concentrations of ethylene glycol and its monoalkyl ethers at 30°C. From it has been calculated the mean slope ($\bar{b}=1.5230$) and the sum of squares for the joint regression (121.781173).

TABLE IIA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 30°C.

Observation	Concentrations of ethylene glycol monomethyl ether			
	35.0 per cent.	37.5 per cent.	40.0 per cent.	42.5 per cent.
$S[(x-\bar{x})(y-\bar{y})]$	4.661846	4.190510	2.212453	3.945919
$S(x-\bar{x})^2$	3.060121	3.194711	1.676468	3.512930
$S(y-\bar{y})^2$	7.476572	5.949571	3.278853	4.811554
N	11	13	7	14
SS for individual regressions . . .	7.255987	5.632065	3.182261	4.686665
b	1.523419	1.311702	1.319711	1.123256
SS pool	7.101944	5.496702	2.919798	4.432276

The analysis of variance of the massed regressions is set out in Table VIII. The z 's for the various combinations have been calculated by the technique employed in Part VI¹ and have been presented in Table IX. The mean square for the residual in y (0.027826) has been used as

TABLE IIB

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 30°C.

Concentration	Item	Sum of squares	N	Mean square
35.0 per cent.	Common regression	7.101944	1	7.101944
	Variation in regression	0.154043	3	0.051348
	Total	7.255987	4	
	Residual in y	0.220585	11	0.020053
37.5 per cent.	Common regression	5.496702	1	5.496702
	Variation in regression	0.135363	3	0.044841
	Total	5.632065	4	
	Residual in y	0.317506	13	0.024431
40.0 per cent.	Common regression	2.919798	1	2.919798
	Variation in regression	0.262463	3	0.087486
	Total	3.182261	4	
	Residual in y	0.096592	7	0.013797
42.5 per cent.	Common regression	4.432276	1	4.432276
	Variation in regression	0.254389	3	0.084796
	Total	4.686665	4	
	Residual in y	0.124889	14	0.008921

denominator to calculate the variance ratios between the different items. The probabilities of the significance between these items have been computed and included in Table IX.

TABLE IIc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 30°C

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
35.0 per cent	0.220585	11	0.154043	3	7.101944	4.661846	3.060121
37.5 per cent	0.317506	13	0.135363	3	5.496702	4.190510	3.194711
40.0 per cent	0.096592	9	0.262463	3	2.919798	2.212453	1.676468
42.5 per cent	0.124889	14	0.254389	3	4.432276	3.945919	3.512930
Totals	0.759572	45	0.806258	12	19.950720	15.010728	11.444230

$$\bar{b} = \frac{15.010728}{11.444230} = 1.311642$$

$$SS \text{ for joint regression} = \frac{(15.010728)^2}{11.444230} = 19.688695$$

TABLE IIId

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOMETHYL ETHER AT 30°C

Item	N	Sum of squares	Mean square
Grand regression	1	19.688695	19.688695
Variation in regression between concentrations	3	0.262025	0.087342
Variation in regression within concentrations	12	0.806258	0.067189
Residual in y	45	0.759572	0.016879

TABLE IIIa

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 30°C

Observation	Concentrations of ethylene glycol monoethyl ether			
	12.5 per cent	15.0 per cent	17.5 per cent	20.0 per cent
$S[(x-\bar{x})(y-\bar{y})]$	5.204340	2.643049	2.917334	2.356392
$S(x-\bar{x})^2$	3.686711	1.924663	2.346900	1.824886
$S(y-\bar{y})^2$	8.478306	4.169134	3.977931	3.174931
N	13	9	11	5
SS for individual regressions	7.867899	3.749695	3.755170	3.122334
b	1.411649	1.173253	1.241059	1.291254
SS pool	7.346699	3.629575	3.626417	3.042701

BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL—PART VII

TABLE IIIb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 30°C.

Concentration	Item	Sum of squares	N	Mean square
12.5 per cent.	Common regression ...	7.346699	1	7.346699
	Variation in regression ...	0.521200	3	0.173733
	Total ...	7.867899	11	
	Residual in y ...	0.610407	13	0.046950
15.0 per cent.	Common regression ...	3.629575	1	3.629375
	Variation in regression ...	0.120120	3	0.040040
	Total ...	3.749695	4	
	Residual in y ...	0.419439	9	0.046604
17.5 per cent.	Common regression ...	3.626417	1	3.626417
	Variation in regression ...	0.128753	3	0.042918
	Total ...	3.755170	4	
	Residual in y ...	0.222761	11	0.020251
20.0 per cent.	Common regression ...	3.042701	1	3.042701
	Variation in regression ...	0.079633	3	0.026544
	Total ...	3.122334	4	
	Residual in y ...	0.052597	5	0.010519

TABLE IIIc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 30°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
12.5 per cent.	0.610407	13	0.521200	3	7.346699	5.204340	3.686711
15.0 per cent.	0.419439	9	0.120120	3	3.629575	2.643049	1.924663
17.5 per cent.	0.222761	11	0.128753	3	3.626417	2.917334	2.346900
20.0 per cent.	0.052597	5	0.079633	3	3.042701	2.356392	1.824886
Totals	1.305204	38	0.849706	12	17.645392	13.121115	9.783160

$$\bar{b} = \frac{13.121115}{9.783160} = 1.341194$$

$$SS \text{ for joint regression} = \frac{(13.121115)^2}{9.783160} = 17.597960$$

TABLE IIIb

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOETHYL ETHER AT 30°C.

Item	N	Sum of squares	Mean square
Grand regression	1	17.597960	17.597960
Variation in regression between concentrations	3	0.047432	0.015811
Variation in regression within concentrations	12	0.849706	0.070809
Residual in y	33	1.305204	0.039348

Test of significance of the difference between the mean squares for variation in regression between concentrations and variations in regression between individual tests.

The same formulas as have been used in Part VI¹ are again used here.

$N_1=19$ and $N_2=73$, therefore $h=30.1522$.

$$\text{Hence } z_{(5 \text{ per cent})} = \frac{1.6449}{\sqrt{30.1522-1}} - 0.7843 \left(\frac{1}{19} - \frac{1}{73} \right) = 0.27416$$

The observed value of $z(1.99119)$ is less than that calculated at the 5 per cent. level, hence $P > 0.05$.

TABLE IVA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 30°C.

Observation	Concentration of ethylene glycol monopropyl ether			
	3 per cent	4 per cent	5 per cent	6 per cent
$S[(x-\bar{x})(y-\bar{y})]$	1.890377	1.439740	2.695830	4.639438
$S(x-\bar{x})^2$	0.897739	0.599865	1.445619	2.113064
$S(y-\bar{y})^2$	4.421353	4.197453	5.949648	11.016602
N	5	5	6	11
SS for individual regressions	4.274127	4.093332	5.760662	10.876641
b	2.105709	2.400107	1.864827	2.195597
SS pool	3.980584	3.455530	5.027254	10.186338

TABLE IVB

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 30°C

Concentration	Item	Sum of squares	N	Mean square
3.0 per cent	Common regression	3.980584	1	3.980584
	Variation in regression	0.293543	2	0.146772
	Total	4.274127	3	
	Residual in y	0.147226	5	0.029445
4.0 per cent	Common regression	3.455530	1	3.455530
	Variation in regression	0.637802	3	0.212601
	Total	4.093332	4	
	Residual in y	0.104121	5	0.020824
5.0 per cent.	Common regression	5.027254	1	5.027254
	Variation in regression	0.733408	3	0.244469
	Total	5.760662	4	
	Residual in y	0.188986	6	0.031498
6.0 per cent	Common regression	10.186338	1	10.186338
	Variation in regression	0.690303	3	0.230101
	Total	10.876641	4	
	Residual in y	0.139961	11	0.012724

BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL—PART VII

Since there was no significant difference between the two items, the sums of squares have been combined and divided by the appropriate number of degrees of freedom to give an error mean square of 0.094755 (Table VIII).

TABLE IVc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 30°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
3.0 per cent.	0.147226	5	0.293543	2	3.980584	1.890377	0.897739
4.0 per cent.	0.104121	5	0.637802	3	3.455530	1.439740	0.599865
5.0 per cent.	0.188986	6	0.733408	3	5.027254	2.695830	1.445619
6.0 per cent.	0.139961	11	0.690303	3	10.186338	4.639438	2.113064
Totals ...	0.580294	27	2.355056	11	22.649706	10.665385	5.056287

$$\bar{b} = \frac{10.665385}{5.056287} = 2.109331$$

$$SS \text{ for joint regression} = \frac{(10.665385)^2}{5.056287} = 22.496832$$

TABLE IVd

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOPROPYL ETHER AT 30°C.

Item	N	Sum of squares	Mean square
Grand regression	1	22.496832	22.496832
Variation in regression between concentrations	3	0.152874	0.050958
Variation in regression within concentrations	11	2.355056	0.214369
Residual in y	27	0.580294	0.021492

TABLE VA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 30°C.

Observation	Concentration of ethylene glycol monobutyl ether			
	1.5 per cent.	2.0 per cent.	2.5 per cent.	3.0 per cent.
$S[(x-\bar{x})(y-\bar{y})]$...	3.109591	3.810624	4.120585	4.262868
$S(x-\bar{x})^2$...	1.924012	2.589282	2.602655	2.416612
$S(y-\bar{y})^2$...	5.471177	6.077422	6.713748	7.853541
N ...	11	11	10	9
SS for individual regressions... ..	5.048367	5.806799	6.591779	7.523255
b ...	1.616202	1.471691	1.583224	1.763985
SS pool ...	5.025726	5.608063	6.523808	7.519636

TABLE Vb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 30°C.

Concentration	Item	Sum of squares	N	Mean square
1.5 per cent.	Common regression ...	5.025726	1	5.025726
	Variation in regression ...	0.022641	3	0.007547
	Total Residual in y ...	5.048367	4	
		0.422810	11	0.038437
2.0 per cent.	Common regression ...	5.608063	1	5.608063
	Variation in regression ...	0.198736	3	0.066268
	Total Residual in y ...	5.806799	4	
		0.270623	11	0.024602
2.5 per cent.	Common regression ...	6.523808	1	6.523808
	Variation in regression ...	0.067971	3	0.022986
	Total Residual in y ...	6.591779	4	
		0.121969	10	0.012197
3.0 per cent.	Common regression ...	7.519636	1	7.519636
	Variation in regression ...	0.003619	3	0.001206
	Total Residual in y ...	7.523255	4	
		0.330286	9	0.036699

TABLE Vc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSION OF CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 30°C.

Concentration	Residual in y		Variation in b		SS pooled b	S[(x- \bar{x})(y- \bar{y})]	S(x- \bar{x}) ²
	SS	N	SS	N			
1.5 per cent.	0.422810	11	0.022641	3	5.025726	3.109591	1.924012
2.0 per cent.	0.270623	11	0.198736	3	5.608063	3.810624	2.589282
2.5 per cent.	0.121969	10	0.067971	3	6.523808	4.120585	2.602655
3.0 per cent.	0.330286	9	0.003619	3	7.519636	4.262868	2.416612
Totals ...	1.145688	41	0.292967	12	24.677233	15.303668	9.532561

$$\bar{b} = \frac{15.303668}{9.532561} = 1.605410$$

$$SS \text{ for joint regression} = \frac{(15.303668)^2}{9.532561} = 24.568660$$

TABLE Vd

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOBUTYL ETHER AT 30°C.

Item	N	Sum of squares	Mean square
Grand regression ...	1	24.568660	24.568660
Variation in regression between concentrations	3	0.108573	0.036191
Variation in regression within concentrations	12	0.292967	0.024414
Residual in y	41	1.145688	0.027944

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Test of significance of the difference between the mean squares for the variation in regression between the different compounds and the residual in y .

Here $N_1=5$ and $N_2=232$; from the statistical tables it is seen that the observed value of z (0.894322) is larger than the theoretical value even at the 0.1 per cent. level, (about 0.7), hence $P < 0.001$.

TABLE VIA

SUMMARY OF TERMS FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSIONS OF DISINFECTION OF *BACT. COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 30°C.

Observation	Concentration of ethylene glycol monohexyl ether				
	0.325 per cent.	0.350 per cent.	0.375 per cent.	0.400 per cent.	0.425 per cent.
$S[(x-\bar{x})(y-\bar{y})]$	2.915882	1.959550	1.374006	2.103304	2.816573
$S(x-\bar{x})^2$	1.219958	0.753519	0.751145	0.987800	1.619699
$S(y-\bar{y})^2$	7.555418	5.700525	3.132374	4.970084	5.323962
N	11	7	8	7	6
SS for individual regressions ...	7.147758	5.402183	2.776206	4.656061	5.117676
b	2.390150	2.600532	1.829215	2.129281	1.738948
SS pool	6.969394	5.095872	2.513353	4.478526	4.897875

TABLE VIb

CALCULATION OF THE ERROR MEAN SQUARE OF REGRESSIONS FROM CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 30°C.

Concentration	Item	Sum of squares	N	Mean square
0.325 per cent.	Common regression	6.969394	1	6.969394
	Variation in regression	0.178364	3	0.059455
	Total	7.147758	4	
	Residual in y	0.407660	11	0.037060
0.350 per cent.	Common regression	5.095872	1	5.095872
	Variation in regression	0.306311	3	0.102104
	Total	5.402183	4	
	Residual in y	0.298342	7	0.042622
0.375 per cent.	Common regression	2.513353	1	2.513353
	Variation in regression	0.262853	3	0.087618
	Total	2.776206	4	
	Residual in y	0.356163	8	0.044521
0.400 per cent.	Common regression	4.478526	1	4.478526
	Variation in regression	0.177535	2	0.088767
	Total	4.656061	3	
	Residual in y	0.314023	7	0.04486
0.425 per cent.	Common regression	4.897875	1	4.897875
	Variation in regression	0.219801	2	0.109901
	Total	5.117676	3	
	Residual in y	0.206286	6	0.034361

TABLE VIc

SUMMARY OF STATISTICAL DATA FROM CALCULATIONS OF PROBIT-LOG. TIME REGRESSION OF CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 30°C.

Concentration	Residual in y		Variation in b		SS pooled b	$S[(x-\bar{x})(y-\bar{y})]$	$S(x-\bar{x})^2$
	SS	N	SS	N			
0.325 per cent.	0.407660	11	0.178364	3	6.969394	2.915882	1.219958
0.350 per cent.	0.298342	7	0.306311	3	5.095872	1.959550	0.753519
0.375 per cent.	0.356168	8	0.262853	3	2.513353	1.374006	0.751148
0.400 per cent.	0.314023	7	0.177535	2	4.478526	2.103304	0.9878
0.425 per cent.	0.206286	6	0.219801	2	4.897875	2.816573	1.6196

$$\bar{b} = \frac{11.169315}{5.332121} = 2.094723$$

$$SS \text{ for joint regression} = \frac{(11.169315)^2}{5.332121} = 23.396618$$

TABLE VIb

MEAN SQUARES OF THE VARIATIONS IN THE PROBIT-LOG. TIME REGRESSIONS FROM DISINFECTATION OF BACT. COLI BY CONCENTRATIONS OF ETHYLENE GLYCOL MONOHEXYL ETHER AT 30°C.

Item	N	Sum of squares	Mean square
Grand regression	1	23.396618	23.396618
Variation in regression between concentrations ...	4	0.558402	0.139605
Variation in regression within concentrations ...	13	1.144864	0.088066
Residual in y	39	1.582479	0.040576

Test of significance of the difference between the mean squares for the variation in regression between concentrations and the residual in y.

Here $N_1=19$ and $N_2=232$, therefore $h=35.1235$; from this $z_{(5 \text{ per cent})} = 0.24366$.

The observed value of $z(0.299278)$ is greater than that calculated at the 5 per cent. level, hence $P < 0.05$.

Test of significance of the difference between the mean squares for the variation in regression between tests and the residual in y.

Here $N_1=73$ and $N_2=232$, therefore $h=111.056$; from this $z_{(5 \text{ per cent})} = 0.1497$.

The observed value of $z(0.308143)$ is greater than that calculated at the 5 per cent. level, hence $P < 0.05$.

Test of significance of the difference between the mean squares for the variation in the pooled regression (i.e. between concentrations between tests and residual in y).

Here $N_1=92$ and $N_2=232$, hence $h=131.7531$; from this $z_{(5 \text{ per cent.})} = 0.1387$.

The observed value of $z(0.3063)$ is greater than that calculated at the 5 per cent. level, hence $P < 0.05$.

Calculation of the standard errors of the probit-log. time regression coefficients at 30°C.

The variance of b is given by the formula $V_b = \frac{V_y}{S(x-\bar{x})^2}$ where V_y is the variance of y (the probit), i.e. the error mean square (0.094755, Table VIII) and $S(x-\bar{x})^2$ is the corrected sum of squares of x (the log).

TABLE VIII

ANALYSIS OF VARIANCE OF MASSED REGRESSIONS FOR DISINFECTION OF *BACT COLI* BY CONCENTRATIONS OF ETHYLENE GLYCOL AND ITS MONALKYL ETHERS AT 30°C

Item	N	SS	Mean square	Variance ratio	Probability
Massed regression	1	121 781173	121 781173		
Variation in regression between compounds	5	4 977121	0 995424		
Variation in regression between concentrations	19	1 750576	0 092136	see Table IX	
Variation in regression between tests	73	6 966892	0 095437		
Residual in y	232	6 455718	0 027826		
Pooled error	92	8 717468	0 094755		

TABLE IX

CALCULATION OF z 's FOR THE ITEMS IN THE ANALYSIS OF VARIANCE OF THE MASSED REGRESSIONS IN TABLE VIII

N_1/N_2	Variance ratio (V R)	$\log_{10} V R$	$\log_e V R = \log_{10} V R \times 1.15129$	$z = \frac{1}{2} \log_e V R$	Probability
19/73	0 965412	1 9847	1 98238	1 99119	> 0 05
5/232	35 773162	1 5536	1 78864	0 89432	< 0 001
19/232	3 311148	0 5199	0 59856	0 29928	< 0 05
73/232	3 429774	0 5353	0 61629	0 30814	< 0 05
92/232	3 405000	0 5321	0 6126	0 3063	< 0 05

TABLE X

THE PROBIT-LOG TIME REGRESSION COEFFICIENTS WITH THEIR STANDARD ERRORS, OF THE REACTION BETWEEN *BACT COLI* AND ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AT 30°C

Compound	b	N	$S(x-\bar{x})^2$	V_y	$s_b = \frac{V_y}{S(x-\bar{x})^2}$	Ratio of b to s_b
Ethylene glycol	1 293798	13	11 356346	0 094755	0 09135	14
Monomethyl ether	1 311642	12	11 444230		0 09099	14
Monoethyl ether	1 341194	12	9 783160		0 09842	14
Monopropyl ether	2 109331	11	5 056287		0 01369	16
Monobutyl ether	1 605410	12	9 532561		0 09978	16
Monohexyl ether	2 094723	13	5 332121		0 13330	16

time) for the regression lines of each compound. The standard error of the regression coefficients (V_b) have been computed and set out in Table X. As in the experiments at 20°C., the ratio of the regression coefficients to their slopes is large, thereby indicating that b has been satisfactorily estimated. Comparison with the results in Part VI, Table IX, indicates that the values of b are higher at 30°C. than at 20°C.

CONCLUSIONS

As with the results from the experiments at 20°C. (Part VI¹), the analysis of variance indicates that there is a significant variation in regressions between the different compounds, i.e. each substance has its characteristic regression coefficient which differs significantly from the average of the series.

The analysis also shows that the variations in the regressions between the concentrations of the substances are of the same order as the variation between the individual tests at a particular concentration. Hence the regressions of different concentrations of the same substance may be taken as parallel.

SUMMARY

1. The course of the disinfection (at 30°C.) between *Bact. coli* and several concentrations of ethylene glycol and the following ethers: monomethyl, monoethyl, monopropyl, monobutyl and monohexyl has been investigated. Several experiments were conducted at every concentration and probit-log. time regressions calculated for all experiments.

2. For every concentration of a substance the sum of squares for the common regression and for the variation in regression were calculated; the error mean square of the regression was also computed.

3. The data for every concentration of each compound have been pooled and a mean regression has been calculated for each compound.

4. The statistical data from all the calculations for the terms of the regressions for every concentration of the compounds (at 30°C.) have been massed and an analysis of variance carried out.

5. The probabilities for the differences between the mean squares of the items in the analysis of variance have been deduced by means of the z distribution.

6. No significant difference could be shown between the variation in regression between concentrations and between tests; these two errors have been pooled in order to establish the error mean square for all the estimations performed.

7. The probit-log. time regression coefficient for every compound has been compared with its standard error; in all cases the ratio was large, thereby indicating that b had been estimated satisfactorily.

REFERENCES

1. Berry and Michaels, *J. Pharm. Pharmacol.*, 1949, 1, 470.
2. Berry and Michaels, *Quart. J. Pharm. Pharmacol.*, 1947, 210, 331.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Cinchonamine, Quinamine and Yohimbol, Relation between. M. Raymond-Hamet. (*C. R. Acad. Sci., Paris*, 1948, 227, 1182.) Cinchonamine has the same empirical formula as yohimbol, the ultra-violet absorption curves are identical, and both give similar colour reactions. On the other hand the different physiological actions of these two compounds suggest a difference in the nuclear skeleton. Cinchonamine only differs from quinamine by having one oxygen atom less, and it has been suggested that its structure only differs by having CH_2OH in place of CH_3 in the α -position on the indole group. Iodomethylation of yohimbine considerably decreases the sympathicolytic activity, whereas with cinchonamine and quinamine it produces a considerable increase in this action. Further the Sivadjan reaction gives quite different results with yohimbine and cinchonamine. In presence of an oxidising agent, cinchonamine gives with sulphuric acid an intense blue colour which is not obtained with quinamine, and is possibly due to the 2:3:4:5-tetrahydro- β -carbolinic grouping. The absorption spectra of cinchonamine and of quinamine are quite different. G. M.

ANALYTICAL

Acetone, Determination of. R. E. Byrne. (*Anal. Chem.*, 1948, 20, 1245.) A method is described which is accurate for amounts of acetone up to 25 $\mu\text{g./ml.}$, being based on the estimation of the hydrochloric acid released after combination of the acetone with hydroxylamine hydrochloride. The release of acid causes a drop in pH and there is a definite relation between pH change and the quantity of acetone present. Standard curves are given connecting pH with acetone content under the prescribed conditions. The effect of ethyl alcohol on the pH is also studied. The range of sensitivity of the curves may be extended by varying the concentration of hydroxylamine hydrochloride used. Determinations are not perceptibly affected by variations of room temperature between 20° and 30°C. R. E. S.

Acetylacetone and Related β -Diketones, Colorimetric Determination of. R. F. Witter, J. Snyder and E. Stotz. (*J. biol. Chem.*, 1948, 176, 493.) Methods are given for the colorimetric determination of 2 to 8 micromoles of a number of β -diketones. The colour reaction involves a condensation of the diketone with α -phenylenediamine in acid solution to produce a reddish purple colour. Acetylacetone in this reaction yields 2:4-dimethyl-1:5-benzodiazapine hydrochloride; triacetic acid appeared to be decarboxylated to acetylacetone during the period of colour development. ethyl triacetate either reacted directly or was partly hydrolysed since the colour per mole of ester was less than with acetylacetone or the free acid: triacetic lactone did not react and was determined after conversion to acetylacetone by hot acid hydrolysis. Detailed conditions for the reaction are given in which the colour developing during 30 minutes at room tem-

perature was determined at 500 $m\mu$ using a photo-electric colorimeter. No colour was formed with diacetyl, acetoin, acetone, acetaldehyde, succinic acid, fumaric acid, acetoacetic acid, oxalacetic acid, lævulinic acid, citric acid, ethyl acetoacetate, ethyl lævulinate, or diethyl acetonedicarbozylate. The following substances interfered with the reaction: tungstic acid, oxidising agents, bisulphite, hydroxylamine, semicarbazide, pyruvic acid, diacetyl, formaldehyde, crotonaldehyde. The crotonaldehyde interference due to the production of a yellow colour with the reagent could be eliminated by the use of sodium bisulphite. A 10 per cent. glucose solution or 0.4 M urea solution did not interfere. The recovery of acetylacetone or triacetic lactone added to various biological systems was of the order of 95 to 100 per cent.

R. E. S.

Adrenaline, Colorimetric Determination of. M. Péronnet. (*Ann. pharm. Franc.* 1948, 6, 365.) In the colorimetric determination of adrenaline, using iodine as oxidant, it is usual to remove the excess of the latter by means of thiosulphate. Since secondary reactions result from the thiosulphate, the author recommends that the excess of iodine be removed by shaking with benzene. The colour obtained in this way is stable over a considerable period, whereas that obtained by the thiosulphate method steadily decreases. Details are as follows. To 5 ml. of a solution of adrenaline 5 ml. of a 10 per cent. solution of sodium acetate and 2 ml. of 0.02N iodine solution are added; after 5 minutes, 2.5 ml. of water is added and the excess of iodine is removed by shaking with three successive 5 ml. quantities of benzene; the colour is then determined.

G. M.

Adrenaline in Pharmaceutical Products, Photometric Determination of. J. R. Doty. (*Anal. Chem.*, 1948, 20, 1166.) The determination depends on the addition of an alkaline buffer to a slightly acid solution containing adrenaline and a ferrous salt, when a blue colour begins to develop at about pH 6.5 and gradually changes to the characteristic red-blue colour which attains a maximum intensity at about pH 8 to pH 8.5. The method is mostly of value when the adrenaline content is at least 10 p.p.m., optical density measurements being made at 530 $m\mu$. The reaction appears to be specific for compounds possessing at least two phenolic groups attached to adjacent carbon atoms. No colour is produced with phenol, neo-synephrine, resorcinol, hydroquinone, orcinol, phloroglucinol, or phthalic acid but an atypical colour response is observed with as little as 5 mg. of salicylic acid. Typical colour reactions are produced by pyrocatechol, pyrogallol, adrenaline and cobefrin. Among inorganic ions bisulphite affects the colour intensity. The method can be used in the presence of procaine hydrochloride without turbidity. Solutions of tetracaine hydrochloride, metycaine hydrochloride and some other products become cloudy or milky when the buffer reagent is added; in this case isopropyl alcohol is added to the anæsthetic solution and the colour is then developed as usual.

R. E. S.

Arsenic, Determination of Small Quantities of. P. Paulssen. (*Pharm. Weekbl.*, 1949, 84, 33.) The method, as applied to arsenic pills, is as follows. 2 pills are rubbed down with a little 6N hydrochloric acid and transferred to a conical flask with 10 to 15 ml. of the acid. 50 mg. of potassium chlorate is added and, after standing for 5 minutes, the mixture is heated on the water-bath for 10 minutes. The mixture is cooled, and the treatment repeated with a further 50 mg. of potassium chlorate. After filtering through a plug of cotton wool, the filtrate is treated with 10 ml. of a 10 per cent.

solution of calcium hypophosphite in 25 per cent. hydrochloric acid, and a little finely divided asbestos is added to the mixture, which is heated on the water-bath for 30 minutes. The precipitated arsenic is filtered off on asbestos supported on a perforated platinum plate, and washed with water, then with alcohol and finally again with water. The plate with the deposit is transferred to a flask, the filter tube being washed with water, and treated with 25 ml. of 0.008N ceric sulphate solution. After the arsenic has completely dissolved, a few drops of a 0.25 per cent. solution of osmic acid in dilute sulphuric acid are added, and one drop of 0.025M o-phenanthroline iron solution, and the excess of ceric sulphate is titrated with 0.005N arsenious acid to the first change to red. The method may also be used for atoxyl and neosalvarsan.

G. M.

***p,p'*-Dichlorodiphenyltrichloroethane (D.D.T., Dicophane) in commercial samples.** R. L. Wain and A. E. Martin. (*Analyst*, 1948, 73, 479.) The determination by the dehydrohalogenation method was studied in detail with particular reference to the behaviour of the *ortho* compound which is the most important impurity in commercial samples. The rates of reaction of samples when treated with standard alcoholic potassium hydroxide solution were determined by estimation of the chloride produced, using Volhard's method. At 23°C. the dehydrohalogenation of both the *o,p'*- and the *p,p'*- compounds was found to be a second-order reaction with rate constants of 0.0008971 and 0.03704 l./mol/sec. respectively. Tables and graphs are given for the rates of dehydrohalogenation of known mixtures of pure *o,p*- and *p,p'* compounds and also for commercial samples of known *p,p'*- content (as determined by Balaban and Calvert's crystallisation method). A reaction time of 60 minutes at 23°C. was chosen for the determination since this effected complete dehydrohalogenation of the *p,p'*- compound while only 0.2 equivalent of chloride ion per mol. was lost from the *o,p'*-isomer. A graph of chloride ion liberated from commercial samples treated under these conditions against *p,p'*-isomer content is given and the following equation: percentage of *p,p*-isomer = $(1.56 \times \text{mg. of Cl}^-/\text{g. of sample}) - 58.1$, is tentatively suggested.

R. E. S.

Phenol and Structurally Related Compounds, Determination of, by the Gibbs Method. M. B. Ettinger and C. C. Ruchhoft. (*Anal. Chem.*, 1948, 20, 1191.) A detailed study is made of the determination of phenols by means of 2:6-dibromoquinone chloroimide. For the determination of phenol in aerobic surface waters, extraction of the colour produced with *n*-butyl alcohol is recommended. The reaction itself should be carried out at room temperature ($\pm 1^\circ\text{C}$.) for both sample and standard at pH 9.4 during 6 to 24 hours. The colour produced differs in the peak light absorption wave-length according to the phenolic compound used and a further difference is found between the value in water and in the *n*-butyl alcohol extract. The following phenols were studied: phenol, *o*-cresol, *m*-cresol, *a*-naphthol, *o*-chlorophenol, *p*-chlorophenol. The colour intensification obtained by *n*-butyl alcohol extraction based upon increase in extinction at the wave lengths of maximum adsorption was 7.3 and 7.5 times for phenol and *o*-cresol, respectively. The limits of linear relationship between the reaction products and the colour formed were found to be up to 0.1 p.p.m. for phenol and at least to 0.3 p.p.m. for *o*-cresol. On the basis of these studies a detailed procedure for phenols and cresols in surface waters, using spectrophotometric examination at the wave-lengths of maximum adsorption, is proposed. An accelerated procedure for obtaining early information, with concurrent

loss of some sensitivity and accuracy, is also given. On waters containing 0.1 p.p.m. of the pollutants the recommended procedure was capable of results with a probable error of only ± 2.3 , ± 1.7 , and ± 2.3 per cent. for one observation for phenol, *o*-cresol, and *m*-cresol, respectively. R. E. S.

ANIMAL SUBSTANCES

Gelatin, Behaviour of Solutions of. G. Rosi and P. M. Strocchi. (*Ann. Chim. appl., Roma*, 1948, 38, 571.) Experiments were made with a sample of gelatin which contained 2.5 per cent of ash (A) and the same sample after partial (B) and complete (C) electrodialysis, which reduced the ash to 0.26 per cent. and 0.05 per cent. respectively, to see what effect the ash had on its behaviour. The pH of the original sample A was 6.76, of B 4.52, and of C 4.65 in a concentration of 0.8065 per cent. Progressive amounts of hydrochloric acid were added, the final amount of 10 ml. of acid to 50 ml. of solution reduced the pH of A to 2.17, B to 2.01 and C to 2.06, intermediate quantities showing intermediate results. Ten solutions of each grade containing 0.4032 per cent. of gelatin were adjusted by the addition of hydrochloric acid or sodium hydroxide to a series of pH between 2 and 7 and tested for conductivity, viscosity and surface tension. The results of the conductivity tests all showed a minimum in the neighbourhood of pH 4.63 and 4.72, the actual figures being highest for A and lowest for C. The curves of viscosity show a maximum between pH 2.85 and 3 and a minimum between pH 4.74 and 4.78. The minimum is more pronounced with greater purity of the gelatin. Surface tensions show a minimum at pH 4.48 for solution A and 4.32 for B and C; solutions B and C show a maximum at pH 2.9 and 3.3. H. D.

GUMS AND RESINS

***Aloe vera*, A Mucilage from.** E. Robez and A. J. Haagen-Smit. (*J. Amer. chem. Soc.*, 1948, 70, 3248.) After drainage of the latex by cutting the leaves near the basal end, the leaves of *Aloe vera* readily yield their mucilaginous layer by scraping after cutting them parallel to the leaf-blade. The mucilaginous parenchyma so obtained, on drying, contains 0.2 per cent. of mucilage and the dried assimilatory tissue 1.73 per cent. Extraction of the ground dried mucilaginous material with alcohol (50 per cent.) and precipitation of the extract by addition of 4 volumes of alcohol (95 per cent.) yielded as precipitate a crude mucilage and a filtrate containing aloin not removed from the leaves by drainage of the latex. The crude mucilage contained 12.9 per cent. of ash which was removed by dialysis from a solution in 0.01N hydrochloric acid against distilled water and the mucilage recovered by precipitation on addition of alcohol. So purified the mucilage was a white amorphous powder decomposing at 271° to 276° C., insoluble in organic solvents, soluble in water to give an optically inactive viscous solution. Elemental analysis indicated the possible composition as a hexosan. With sulphuric acid, the mucilage yielded a hydrolysate free from ketoses, and containing 89 per cent. of aldoses (equal parts of mannose and glucose) and 2.37 per cent. of uronic acid. F. H.

Carob Gum, Constitution of. F. Smith. (*J. Amer. chem. Soc.*, 1948, 70, 3249.) Carob gum, a polysaccharide obtained from the carob bean (*Ceratonia Siliqua* L.) by extraction of the seeds with water or aqueous

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alkaline solution, is shown to be composed of D-galactose (20 per cent.) and D-mannose (80 per cent.). The methylated gum obtainable either directly or through the acetate by treatment with methyl sulphate and sodium acetate, yielded on methanolysis the glycosides of 2:3:4:6-tetramethyl-D-galactose (1 part), 2:3:6-trimethyl-D-mannose (2 to 3 parts) and 2:3-dimethyl-D-mannose (1 part), identified by formation of the crystalline derivatives. The structure of the galacto-mannan polysaccharide is discussed.

F. H.

ORGANIC CHEMISTRY

Pteridine, A Synthesis of. W. G. M. Jones. (*Nature*, 1948, 162, 524.) 2-Chloro-4-amino-5-nitropyrimidine, m.pt. 232°C. is hydrogenated in methyl alcohol over a nickel catalyst to give 2-chloro-4:5-diaminopyrimidine, m.pt. 232°C. which is dehalogenated catalytically over palladium on charcoal in the presence of barium oxide to give 4:5-diaminopyrimidine, m.pt. 204°C. (nitrate decomposes without melting above 260°C.) Reaction of the diaminopyrimidine in aqueous solution with glyoxal bisulphite gave pteridine, crystallizing from alcohol in pale yellow plates, m.pt. 140°C. Pteridine was soluble in water and alcohol and readily sublimed *in vacuo*. The ultra-violet absorption spectrum in aqueous solution at pH 5.8 showed a sharp maximum at $299m\mu, \epsilon = 7,890$, with a violet-blue fluorescence in the ultra-violet in neutral or alkaline solution. It formed a picrate, m.pt. 117.5°C. and an oxalate which decomposed without melting above 128°C.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Benzylpenicillanic Acid, a Crystalline Form of. N. R. Trenner and R. P. Buhs. (*J. Amer. chem. Soc.*, 1948, 70, 2897.) The free acid, benzylpenicillanic acid, associated with one molecular proportion of di-isopropyl ether of crystallisation, is obtainable in crystalline form by extraction of an aqueous solution of sodium benzylpenicillinate (sodium penicillin G) at pH 2.5 with di-isopropyl ether, drying the ether phase over anhydrous sodium sulphate and allowing evaporation at room temperature. This procedure enabled crystalline benzylpenicillanic acid di-isopropyl etherate to be separated from other penicillins and from a crude penicillin sodium salt. Crystalline benzylpenicillanic acid di-isopropyl etherate was found to retain its stability over long periods even when exposed to ordinary laboratory air. It was not hygroscopic and is considered to be superior to any other form of benzylpenicillin (penicillin G) as a primary standard of purity. Dissolved in a pH 7 phosphate buffer it contained 1420 units/mg. when assayed by the cup-plate method against *Staphylococcus aureus*, this activity being equivalent to 1740 units/mg. in the sodium salt. The optical activity, equivalent weight potentiometrically, ultra-violet and infra-red spectra were examined and are discussed as evidence of the purity and nature of the crystalline material. That the di-isopropyl ether merely permits the formation of a well-defined crystal lattice and so is present as a simple solvate of crystallisation and does not form an oxonium salt with benzylpenicillin is supported by the inability to induce crystallisation of benzylpenicillanic acid in the presence of a wide range of other ethers.

F. H.

N-Methylvaline, Optical Resolution of, A New Phenomenon. A. H. Cook, S. F. Cox and T. H. Farmer. (*Nature*, 1948, 162, 61.) A new and simple

method of resolution of N-methylvaline has been found in connection with the study of the nature and configuration of hydrolytic products of the antibiotic lateritium-I derived from *Fusarium lateritium*. It was found that the lactone derived from D- α -hydroxyisovaleryl-D-N-methylvaline is more strongly adsorbed on an acid-washed alumina column than that derived from D- α -hydroxyisovaleryl-N-methylvaline and undergoes hydrolysis. Laevo- α -Bromisovaleryl chloride and DL-N-methylvaline were condensed together and the product was refluxed with pyridine and water when the hydroxy-acid condensation product was formed. It was extracted and heated for several hours on a steam-bath under reduced pressure to yield a lactone having $[\alpha]_D^{25} + 52^\circ$ in alcohol. On passing an ethereal solution of the lactone through an acid-washed alumina column about half the material was lost. The remainder had $[\alpha]_D^{25} - 79^\circ$ in alcohol and on hydrolysis yielded L(-)-N-methylvaline.

F. H.

Penicillins, Synthetic, Preparation and Antibacterial Properties of Crude Sodium Salts. F. H. Carpenter, G. W. Stacey, D. S. Genghof, A. H. Livermore and V. du Vigneaud. (*J. biol. Chem.*, 1948, 176, 915.) Minute yields of crude sodium salts of several penicillins were obtained by synthesis. In the reaction used, appropriate oxazolones were condensed with α -amino- β -mercapto acids to yield penicillenic acids which were isolated as amorphous solids characterised by their ultra-violet absorption spectra. The penicillenic acids were converted in small yield to the corresponding penicillins, the reaction conditions depending on the type of acid involved. In this manner benzylpenicillin analogues in which the D-penicillamine fraction was replaced by two isomeric DL- β -methyl cysteines, by DL- β , β -diethylcysteine, or by DL- β -ethyl- β -methyl cysteine were prepared. In addition, crude sodium D-phenylpenicillin and D-styrylpenicillin were synthesised. Antibiotic activities against *Staphylococcus aureus* H, *Bacillus subtilis*, and *Vibrio metchnikovii* were determined. Qualitative tests showed that the crude synthetic penicillins did not possess marked antibacterial properties against *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Proteus vulgaris* OX-19, or *Pseudomonas aeruginosa*, organisms which also showed a high degree of resistance to the action of crystalline sodium benzylpenicillin.

R. E. S.

Pituitary, Anterior, Crystalline Growth Hormone, A New Preparation of. A. E. Wilhelmi, J. B. Fishman and J. A. Russell. (*J. biol. Chem.*, 1948, 176, 735.) A new method is described for the preparation of crystalline growth hormone from fresh bovine anterior pituitary glands. Fractionation of calcium hydroxide extracts of the ground glands, using ethyl alcohol at low temperatures following the method of Cohn *et al.* (*J. Amer. chem. Soc.*, 1946, 68, 459) for the separation of plasma proteins, yielded an abundance of crude fractions with high growth-promoting activity. The active fractions were redissolved in dilute potassium chloride solution followed by removal of the bulk of impurities in a precipitate formed at pH 5.0 and by a fractional precipitation with ethyl alcohol from pH 8.5 to 8.7, when a crystalline protein was obtained, electrophoretically homogeneous, which from its biological activity and other properties was identified as the anterior pituitary growth hormone. The yield of the crude primary fractions averaged about 33 g./kg. of fresh glands, with a corresponding yield of the order of 3 g. of pure crystalline growth hormone. The pure crystalline

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product had the expected effects upon body growth and upon the width of the proximal epiphyseal cartilage of the tibia in hypophysectomised rats. It was also the most active glycostatic hormone preparation yet isolated.

R. E. S.

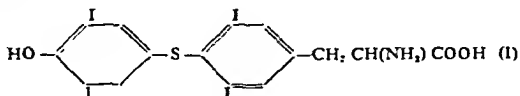
Riboflavine and Allied Substances, Production during the Growth of *Corynebacterium diphtheriae*. A. J. Wo i w o d and F. V. Ling g o o d. (*Nature*, 1948, 162, 219.) The report of Wadsworth and Crowe (*J. Infect. Dis.*, 1943, 73, 106) that a special strain of *C. diphtheriae*, grown on a synthetic medium, elaborates a flavine, has been confirmed. Park Williams No. 8 strain of *C. diphtheriae*, grown on casein hydrolysate produced a flavine which gave two fluorescent spots on a paper partition chromatogram, using *n*-butyl alcohol-acetic acid as a solvent. Evidence has been produced that the faster-running spot was riboflavine, and it has been suggested that the slower running spot was either riboflavine phosphate or flavine adenine dinucleotide.

F. H.

Sodium Ascorbate, Crystalline Form of. S. L. Ruskin and A. T. Merrill. (*Science*, 1948, 108, 713.) A stable sodium ascorbate is obtained by the reaction of ascorbic acid with sodium methylate in methyl alcohol. The product was more stable than ascorbic acid itself and showed no decline in potency after 500 hours at 45°C. in closed glass containers. For the preparation 88 g. of ascorbic acid was dissolved in 600 ml. of hot absolute methyl alcohol. While still hot, it was treated under stirring with 250 ml. of a warm solution of sodium methylate containing 12.5 g. of sodium. The combined solutions were stirred until the resulting precipitate of sodium ascorbate crystallised (about 15 minutes); the sodium ascorbate was then filtered with suction and washed with a little methyl alcohol. It could be dried *in vacuo* at a temperature as high as 100°C. producing a 95 per cent. yield of pure product. Analysis indicated the presence of about 1 per cent. of water; the rotation was + 102.99° and the assay by iodine titration gave a result of 87.55 per cent. (theory 88.9). Further procedures were developed using sodium hydroxide, sodium carbonate, or sodium hydride with similar results. Petrographic studies showed the variety of crystallisations occurring and, together with the nature of the hydroxyl group reaction during neutralisation, provided evidence suggesting a possible explanation of the stability of the sodium ascorbate crystals prepared.

R. E. S.

Thyroxine, Synthesis of a Sulphur-containing Analogue of. C. R. H a r i n g t o n. (*Biochem. J.* 1948, 43, 434.) The possibility of controlling thyroid function by means of compounds inhibiting the output of thyrotrophic hormone from the anterior pituitary gland is discussed. The compound (I) which is identical with thyroxine save that sulphur replaces oxygen, was selected for trial.



An attempt was made to synthesise the compound on the lines of thyroxine itself and by starting with 4-methoxythiophenol and 3:4:5-triiodonitrobenzene, 3:5-diiodo-4(4'-methoxy)phenylsulphidobenzaldehyde was obtained by a series of reactions similar to those employed in the thyroxine synthesis. At this stage the modified Erlenmeyer method did not yield

3:5-diiodo-4(4'-hydroxy)phenylsulphidophenylalanine and a different route had to be used. The aldehyde was reduced to the corresponding alcohol by means of aluminium isopropoxide; the alcohol was converted into the chloride and the latter condensed with ethyl acetamidomalonate; appropriate treatment of the resulting ester with a mixture of hydrobromic and acetic acids gave the hydroxydiiodoamino-acid in satisfactory yield and final iodination in ammoniacal solution afforded the desired thyroxine analogue. In acute experiments the thyroxine analogue was non-toxic to mice in doses up to 0.5 g./kg. (subcutaneous) and 0.25 g./kg. (intravenous). Tested on tadpoles (*Xenopus levis*) it accelerated metamorphosis, its activity in this respect being approximately one-fifth of that of thyroxine.

R. E. S.

Vitamin B₁₂ Group of Factors, Chromatography of. W. F. J. Cuthbertson and E. L. Smith. (*Biochem. J.* 1949, 44, v.) A combination of partition chromatography on paper with microbiological assay on a solid medium enables the two red clinically active substances, thymidine, and a fourth microbiologically active component present in liver extract to be demonstrated. Water-saturated *n*-butyl alcohol is used, with upward or downward development, two techniques being available for observing the developed spots. A drop of adequately purified material, containing at least 10 μ g. of the factors, gives directly visible red spots. The crystalline factor gives a single spot, but the mother liquors usually give a second fainter and slower-moving spot and occasionally a third, which travels fastest and appears to be a microbiologically inactive degradation product. Fairly crude extracts can be used for the other technique since only 0.005 and 0.1 μ g. of the factors are required: the developed strip is applied to the surface of nutrient agar seeded with *Lactobacillus lactis* Dorner, being removed after 10 min. and the plate incubated overnight. The usual pattern is an ellipse of growth not far from the origin and another a few cm. along, while much farther along, and beyond the position occupied by riboflavine (if present), are one or two zones of attenuated growth: the first two zones are due to the red factors, the third to a substance not yet characterised, and the fourth to thymidine. Samples of the 'animal protein factor' of bacterial origin were examined by this technique and two zones of strong growth and one or two of diffuse growth were always observed, these being always in the same relative positions as the zones from liver extract. It is concluded that the bacteria elaborate the anti-pernicious anæmia factor and probably also the second active red factor, as well as thymidine.

R. E. S.

BIOCHEMICAL ANALYSIS

Aerial Bactericides, Evaluation of. (*Chem. Ind.*, 1949, 68, 115.) This report of the Aerosols Panel of the British Disinfectant Manufacturers' Association proposes that aerosols and other gaseous products for which bactericidal claims are made should be recommended for use at such concentrations as are capable of reducing the bacterial content of the test chamber by not less than 85 per cent. when tested by the technique described. The test chamber is preferably cubical, of a capacity of 500 to 1,000 cu. ft., and suitably equipped with a filter for incoming air, fans for mixing the air and for ventilation, and an arrangement for sampling the air. The procedure is to count the normal bacterial population of the chamber, to spray it with a culture of the test organism and count again, and finally to disperse the

bactericidal agent and determine the percentage survival in the period 4 to 6 minutes from the commencement of the dispersal of the bactericidal agent. The whole procedure is carried out at a temperature of $20 \pm 3^\circ\text{C}.$, and a relative humidity of 60 per cent. ± 5 per cent. The test organism is a special non-pathogenic strain of *Staphylococcus albus* deposited at the National Collection of Type Cultures under the name *Staphylococcus albus*. Aerosols strain. It is grown on a special liquid culture medium and the culture is diluted with broth for spraying; for the production of the spray an M.A.3 Aerolyser Spray Assembly is used. The bactericidal agent is dispersed from an electrically heated boat of nickel-chrome alloy. The air in the chamber is sampled by means of a slit sampler and petri dishes containing a special solid culture medium and the plates are incubated for 24 hours at $37^\circ\text{C}.$ before counting. To enable different workers to ensure that the strain of bacteria they are using is exhibiting normal resistivity and also to indicate the degree of reproducibility being attained by different laboratories, cyclo-pentanol-1-carboxylic acid is recommended as a standard reference bactericide. The technique described is also suitable as a preliminary sorting test for potential bactericides and for comparing the efficiencies of apparatus designed to disperse aerial bactericides.

G. R. K.

Calciferol, Colorimetric Determination of. P. B. Nielsen (*Dansk Tidsskr. farm.*, 1949, 23, 21.) Calciferol may be determined colorimetrically within less than 3 per cent. by means of the antimony chloride reaction, provided alcohol is absent. Ordinary chloroform is purified by washing 3 times with water, drying over sodium sulphate, and fractionating, rejecting the first and last tenth. The middle fraction is collected over sodium sulphate. It should be tested for freedom from chlorides, oxidising agents (potassium iodide-starch) and alcohol—the latter by shaking 5 ml. with 20 ml. of water, and adding to 10 ml. of the aqueous solution 2 drops of 1.7 per cent. potassium dichromate solution and 5 ml. of concentrated sulphuric acid. The yellow colour of the solution should not change within 5 minutes. The reagent is prepared by dissolving 100 g. of antimony chloride in chloroform to 500 ml., filtering, and adding 10 g. of redistilled acetyl chloride. A quantity, up to 1 ml., of the solution to be tested, is treated with 3 or 4 ml. of the reagent, and the extinction (using filter S50) is measured within 1 to 2 minutes. For crystalline calciferol the extinction $E_{1\text{ cm.}}^{1\text{ per cent.}}$ at 500 m μ is about 1700. Solutions in arachis oil can be assayed directly, without saponification, down to 75,000 I.U./ml., but at lower concentrations allowance must be made for the absorption of the oil itself, determined in a similar manner. Solutions in alcohol may be assayed as follows: a suitable quantity of the solution is weighed into a 50-ml. measuring flask, 50 cg. of arachis oil is added, and carbon dioxide, previously washed with concentrated sulphuric acid, is passed through the flask. The oil should be clear within 45 minutes. The glass tube and neck of the flask is washed down with a little purified chloroform, and the gas is again passed for 30 minutes. The washing is repeated and the flask is filled up with purified chloroform, the test being then continued as before. A solution of calciferol in alcohol (96 per cent.), of 300,000 I.U./g., was found to be unchanged in strength after 14 months.

G. M.

Caronamide, Colorimetric Determination of. C. Ziegler and J. M. Sprague. (*J. Lab. clin. Med.*, 1948, 35, 96.) Caronamide (4'-carboxy-

phenylmethanesulphonanilide, $C_6H_5.CH_2.SO_2NH.C_6H_4.COOH$) was found to be resistant to hydrolytic cleavage except under conditions that resulted in the destruction of the *p*-aminobenzoic acid produced. The action of Raney catalyst (powdered nickel aluminium alloy) in alkaline solution, however, gave complete cleavage and the *p*-aminobenzoic acid could be determined by established procedures. Details are given for the determination of caronamide in water, urine, plasma and blood, using this process. In the procedures for plasma and blood, alcohol (90 per cent.) was used as a protein precipitant; in an alternative method, plasma in alkaline solution was treated directly with the alloy. The liberated *p*-aminobenzoic acid was determined colorimetrically by diazotisation using *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Results obtained from aqueous solutions of *p*-aminobenzoic acid of known concentration showed that the alloy treatment caused a loss of from 10 to 20 per cent. of this compound although the recoveries at this level were fairly constant and reproducible. Since recovery experiments on caronamide itself gave losses of a similar order it appeared that the caronamide was split quantitatively but that an average of 15 per cent. of the liberated *p*-aminobenzoic acid was either destroyed or lost by adsorption on the finely divided nickel. The use of standard reference curves, prepared from readings of the depth of colour obtained when known amounts of caronamide in urine, plasma, or blood were subjected to the method, is therefore necessary.

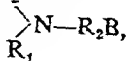
R. E. S

CHEMOTHERAPY

***β*-Aminoethyl Heterocyclic Nitrogen Compounds, Histamine Activity of.** H. M. Lee and R. J. Jones (*J. Pharmacol.* 1949, 95, 71.) A series of 24 *β*-aminoethyl heterocyclic nitrogen compounds was tested for histamine activity in the hope that some correlations between chemical constitution and histamine activity might be drawn. Eleven of the compounds were found to be active. The one structural feature common to all of the active compounds tested is the system in which the portion $-N=\overset{\overset{|}{\text{C}}}-$ or $=N-\overset{\overset{||}{\text{C}}}-$ is part of an aromatic nucleus, $-N=\overset{\overset{||}{\text{C}}}-CH_2CH_2NH_2$ or $=N-\overset{\overset{||}{\text{C}}}-CH_2CH_2NH_2$. The presence of this structural fragment does not, however, guarantee that a compound will have histamine activity, most of the inactive compounds also have the system in their structures. The size and shape of the aromatic nucleus appears to have a definite bearing on the activity of the compounds. Substitution by a methyl group in the 2- position of histamine lowered the activity several-fold, but similar substitution in the 1- position lowered the activity several hundred fold. The spatial disposition of the various atoms in the aromatic ring, and the nature of the atoms themselves, have a profound influence on the histamine activity. The authors concluded that from this series of compounds no correlations could be drawn between histamine activity and the intrinsic chemical properties of the aromatic nuclei.

S. I. W.

Diamines, 2-Thenyl Substituted, with Antihistamine Activity. F. Leonard and U. V. Solmsen. (*J. Amer. chem. Soc.*, 1948, 70, 2064) A series of 6 new and 2 previously reported 2-thenyl substituted alkylene-diamines which conform to the general type $C_4H_9SCH_2$



where R_1 is phenyl or 2-pyridyl, R_2 is a straight or branched alkylene chain containing two or three carbon atoms and B is a dimethylamino or piperidine group, were prepared. Intermediate secondary amines R_1NHR_2B (I) and $R_1NHCH_2C_4H_9S$ (II) were prepared by condensing aniline with a dialkylaminoalkyl chloride hydrochloride or 2-thenyl chloride, and 2-aminopyridine with dialkylaminoalkyl chlorides or 2-thiophenealdehyde followed by reduction. The tertiary amines were obtained by the alkylation of the intermediates (I) and (II) in benzene or toluene solution in the presence of sodamide. These were converted to water-soluble salts in which form they were evaluated pharmacologically. Preliminary results showed that several of the compounds possessed antihistamine activity similar to that of currently available substances. N,N -dimethyl- N' -phenyl- N' -(2-thenyl)-ethylenediamine was characterised by a very low toxicity and a high order of activity. Attempts to form either hydrochlorides or hydrobromides of N_2,N_2 -dimethyl- N_1 -phenyl- N_1 -(2-thenyl)-1:2-propanediamine and N_2,N_2 -dimethyl- N_1 -(2-pyridyl)- N_1 -(2-thenyl)-1:2-propanediamine resulted in their decomposition. The decomposition of N_2,N_2 -dimethyl- N_1 -phenyl- N_1 -(2-thenyl)-1,2-propanediamine under conditions of hydrochloride formation, observed after the removal of solvents from the neutralisation mixture was investigated in some detail; breakdown products were identified and a mechanism of the procedure suggested.

R. E. S.

Sulphadiazine and Paludrine, Resistance of the Malaria Parasite of the Fowl (*Plasmodium gallinaceum*) to. A. Bishop and E. W. McConnachie. (*Nature*, 1948, 162, 541.) Treatment with gradually increasing doses of sulphadiazine yielded a strain of *Plasmodium gallinaceum* in young chicks in which the resistance to the drug is 32 times greater than that of the untreated strain. Whereas the minimum dose of drug which produces an adverse effect upon the growth-rate of the normal strain, when given twice daily on successive days, is 0.625 mg./20 g. of body-weight, in the resistant strain growth of the parasites occurs in birds receiving 20 mg. twice daily. The production of this 32-fold resistance to sulphadiazine took approximately 12 months. The degree of resistance acquired has been assessed at intervals, by comparing the intensities of infections produced by standard inocula (that is 50,000,000 parasites per chick intravenously) of (a), the resistant strain, into groups of chicks receiving doses ranging from 2.5 to 20 mg. of sulphadiazine twice daily for 3½ days, and (b), the normal parent strain, into groups of chicks receiving similar drug treatment. The sulphadiazine-resistant strain produced infections in chicks receiving doses of drug which inhibited the development of the normal strain (that is, 2.5 to 20 mg.); but the resulting infections were not as heavy as those produced by the parent strain in untreated birds. The sulphadiazine-resistant strain produced, on the whole, less intense infections in untreated birds than did the parent strain. In the sulphadiazine-resistant strain of *P. gallinaceum*, exoerythrocytic schizonts were found in chicks during treatment with this drug. This sulphadiazine-resistant strain of *P. gallinaceum* was also resistant to sulphathiazole, sulphanilamide and sulphapyridine. The cross resistance to sulphadiazine and paludrine was also studied; the sulphadiazine resistant strain of *P. gallinaceum*, when tested by the method described was found to be resistant to 0.1 mg. of paludrine although it had not been subjected to treatment with that drug. Since acquired resistance to sulphadiazine was found to confer resistance to paludrine, the effect of sulphadiazine upon a paludrine-resistant strain of *P. gallinaceum* was studied; the strain was found

to be resistant to doses of 1.25 mg. of sulphadiazine. In one strain tested the development of resistance to paludrine was not accompanied by resistance to sulphadiazine. Various aspects of the drug-resistance are discussed.

R. E. S.

PHARMACY

GALENICAL PHARMACY

Isotonic Solutions, Preparation of. C. G. Lund, K. Pedersen-Bjergaard and E. B. Rasmussen. (*Dansk. Tidsskr. Farm.*, 1949, 23, 119.) The apparatus used for the micro determination of osmotic pressure consists essentially of a short length of constantin wire, bent into an inverted U, and soldered at each end to a manganin wire. The junctions are formed into small loops, each capable of holding about 0.01 ml. of liquid. This apparatus is used as a delicate thermocouple to determine difference of temperature between two drops of liquid. A drop of liquid under examination is placed on one loop, and a drop of sodium chloride solution, of suitable concentration, on the other one. The apparatus, enclosed in a suitable vessel, is kept in a constant temperature bath, and, after 10 minutes, the potential difference is noted with the aid of a sensitive galvanometer. By adjustment of the concentrations, it is possible to find the concentration of a sodium chloride solution which has the same osmotic pressure or depression of freezing point as the solution under examination. The table summarises the results.

Substance	Concentration per cent.	Depression of freezing-point
Sodium chloride	0.9	0.52
Bensulphamide hydrochloride	3.72	0.52
Calcium lævulnate (2H ₂ O)	3.57	0.52
Histidine hydrochloride (H ₂ O)	3.44	0.52
Neostigmine bromide	4.95	0.52
Pethidine hydrochloride	4.79	0.52
Pilocarpine nitrate	4.62	0.52
Sodium acetate (3H ₂ O)	2.03	0.52
Sodium metabisulphite	1.38	0.52
Sulphadiazine sodium	4.24	0.52
Sulphamerazine sodium	4.53	0.52
Sulphathiazole sodium (1½H ₂ O)	4.82	0.52
Cetyltrimethylammonium bromide	5.0	0.233
Morphine sulphate (5H ₂ O)	6.0	0.298
Oxyquinoline sulphate	8.0	0.441
Strychnine hydrochloride (2H ₂ O)	2.5	0.191
Tubocurarine hydrochloride	5.0	0.271

G. M.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and norAdrenaline Injections, and Further Studies on Liver Sympathin. G. B. West. (*Brit. J. Pharmacol.*, 1948, 3, 189.) Examination of the pressor effects of *dl*-noradrenaline and *l*-adrenaline, when injected into the jugular, femoral and splenic veins, and the splenic and external iliac arteries, of cats and rabbits, showed both substances to be less active by portal

where R_1 is phenyl or 2-pyridyl, R_2 is a straight or branched alkylene chain containing two or three carbon atoms and B is a dimethylamino or piperidine group, were prepared. Intermediate secondary amines R_1NHR_2B (I) and $R_1NHCH_2C_4H_8S$ (II) were prepared by condensing aniline with a dialkylaminoalkyl chloride hydrochloride or 2-thenyl chloride, and 2-aminopyridine with dialkylaminoalkyl chlorides or 2-thiophenealdehyde followed by reduction. The tertiary amines were obtained by the alkylation of the intermediates (I) and (II) in benzene or toluene solution in the presence of sodamide. These were converted to water-soluble salts in which form they were evaluated pharmacologically. Preliminary results showed that several of the compounds possessed antihistamine activity similar to that of currently available substances. N,N -dimethyl- N' -phenyl- N' -(2-thenyl)-ethylenediamine was characterised by a very low toxicity and a high order of activity. Attempts to form either hydrochlorides or hydrobromides of N_2,N_2 -dimethyl- N_1 -phenyl- N_1 -(2-thenyl)-1:2-propanediamine and N_2,N_2 -dimethyl- N_1 -(2-pyridyl)- N_1 -(2-thenyl)-1:2-propanediamine resulted in their decomposition. The decomposition of N_2,N_2 -dimethyl- N_1 -phenyl- N_1 -(2-thenyl)-1,2-propanediamine under conditions of hydrochloride formation, observed after the removal of solvents from the neutralisation mixture was investigated in some detail; breakdown products were identified and a mechanism of the procedure suggested.

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CHEMOTHERAPY

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than by jugular vein, though whereas with adrenaline the ratio value for equipressor doses by these routes decreased as the pressure rise increased, with noradrenaline the ratio value remained constant. Unlike adrenaline, noradrenaline when injected into the portal circulation is not potentiated by the simultaneous administration of guanidine or cocaine, and is therefore not rapidly absorbed from the blood stream during its passage through the liver. Both adrenaline and noradrenaline by intra-arterial and intrajugular injection failed to show potentiation by the simultaneous administration of guanidine by the same routes, though both were enhanced by cocaine. Experiments to obtain further evidence of the similarity between intraportal injections of noradrenaline and hepatic nerve stimulation, showed that hepatic nerve stimulation and small intraportal doses of noradrenaline both produced depressor responses, while corresponding doses of adrenaline were without effect; guanidine and cocaine by intraportal injection were also found not to potentiate the action of liver sympathin. When injected into the artery supplying the caudal end of the spleen, adrenaline in small doses caused a small rise followed by a large fall in blood pressure, the depressor effect being possibly due to the liberation of histamine; noradrenaline, on the other hand, produced a pure rise of blood pressure at all dose levels. Noradrenaline is therefore a much more potent pressor agent by splenic artery than adrenaline, and, in addition, relatively more adrenaline than noradrenaline is inactivated in the spleen.

S. L. W.

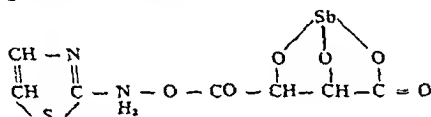
Amidone, Pharmacology of the Optical Isomers of. R. H. Thorp. (*Brit. J. Pharmacol.*, 1949, 4, 98.) The relative analgesic properties of *d*-, *l*-, and *dl*-amidone were determined on rats in comparison with that of morphine; *dl*-amidone hydrochloride was shown to be a rather more powerful analgesic than morphine sulphate. The activity of the *laevo* isomer was very much greater than that of the racemic form, which failed to produce a graded increase in pain threshold when given in large doses. The acute toxicity for the three isomers is approximately equal, and is due to direct action on the cardiac muscle; it is increased with the *l*- or *dl*-isomers by the central nervous depression which these two drugs produce. The depressant effect of *l*-amidone on rabbit respiration is twice as great as that of the racemic compound, the effect of the *dextro*-isomer being negligible in comparable doses. Local anæsthetic action is greatly influenced by the optical isomerism; it is shown by all three isomers, but is greatest in the *laevo* isomer which is 3.5 times as potent as procaine. Amidone and its optical isomers are rather more active than pethidine as spasmolytic drugs; the effect is not associated with the optically active carbon atom but is a function of the molecule as a whole. The reported property of analgesic drugs of producing a state of "acute vascular tolerance" to the depressor action resulting from intravenous injection was confirmed with *l*-amidone.

S. L. W.

Antihistamine Drugs, Pharmacology of. A. M. Lands, J. O. Hoppe, O. H. Siegmund and F. P. Luduena. (*J. Pharmacol.*, 1949, 95, 45.) This is a report of an investigation on the antihistamine drugs *N*'-(2-pyridyl)-*N*'-(3-thenyl)-*N,N*-dimethylethylene-diamine (WIN 2848), *N*'-(2-pyridyl)-*N*'-(2-chloro-3-thenyl)-*N,N*-dimethylethylene-diamine (WIN 2875) and *N*'-(2-pyridyl)-*N*'-(2-bromo-3-thenyl)-*N,N*-dimethylethylene-diamine (WIN 2876). WIN 2848 was shown to be a highly active histamine antagonist, and was potent in this respect than WIN 2875 and WIN 2876. In a dose of 0.013 mg./kg. it protected guinea-pigs against 2.8 intravenous lethal doses

of histamine: a dose of 0.028 mg./kg. of pyribenzamine was required to give the same protection. The three WIN compounds appear to be of the same order of toxicity as pyribenzamine when administered intravenously in mice. WIN 2848 was shown to diminish markedly the severity of the effects of histamine given percutaneously in man. It is suggested that WIN 2848 may be found useful in the treatment of allergic conditions. S. L. W.

Antimonyl 2-Aminothiazole Tartrate, Preparation and Toxicity of. D. B. Meyers and J. W. Jones. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 41.) An organic compound of antimony which might prove highly toxic to protozoa in quantities safe for administration to the host was required. Properties desired were: solubility in water so that parenteral administration was possible; a low index of irritation at the site of injection; a high parasiticidal action; and a sufficient margin of safety. The compound prepared was a water-soluble trivalent antimony salt, which was found to be less toxic and less irritating than tartar emetic when injected intravenously into the femoral vein of white rats. Analyses for antimony and sulphur indicated the following structural formula:



The LD₅₀ of the compound for rats was shown to be 75 to 95 mg./kg.

G. R. K.

Decamethonium Iodide (C10), Effects of on Respiration and on Induced Convulsions in Man. D. L. Davies and A. Lewis. (*Lancet*, 1949, 256, 775.) From a study of the effects of C10 and *d*-tubocurarine chloride in 18 patients with depressive illness subjected to electrically induced convulsions, no distinguishable difference was observed, either in respect of the pareses and paralyses induced or the characteristics of the modified convulsion. The effect of the two drugs on respiratory movement showed no significant difference. Small doses of either produced thoracic movements of lesser amplitude, with slight increase in the rate but no change in the amplitude of abdominal movements. Large doses abolished thoracic movement and diminished the amplitude of abdominal movements, while greatly increasing their rate. Although the efficiency of respiration is impaired by both drugs in large doses C10 spares the diaphragm more than does *d*-tubocurarine chloride, and would thus appear to offer a wider margin of safety. The clinical use of C10, in combination with thiopentone sodium, in a series of 40 patients who were being treated by electrically induced convulsions, showed it to be safe and effective for this purpose and without disagreeable side-effects.

S. L. W.

Digitalis Products, The Use of Pigeons for the Assay of. A. Lavallee and M. G. Allmark. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 45.) Comparative data are given on the intravenous pigeon and U.S.P. cat methods for powdered digitalis, digitalis tinctures, tablets and capsules, purified glycosidal products and amorphous digitalin. The pigeon method used has been described by Cook (*Bull. U.S.P.*, 1948, 35, 89.), and by Braun and Lusk (*J. Pharmacol.*, 1948, 93, 81.) The two methods showed fairly

[Continued on page 635]

BOOK REVIEWS

THE CHEMISTRY OF PENICILLIN, edited by Hans T. Clarke, John R. Johnson and Sir Robert Robinson. Pp. 1042 and Appendix. Princeton University Press, New Jersey (London: Geoffrey Cumberlege) 1949, £9 9s. 0d.

Many of the successful results achieved by scientific workers during the war are now known to the general public, and many are aware that chemists in this country and the U.S.A. working together under the auspices of the Medical Research Council and the Office of Scientific Research and Development respectively, determined the probable constitution of penicillin but failed to discover a commercial method of synthesising it. Because the results were not of immediate practical value, there may have been a tendency to dismiss them as of little importance and to regard the effort and money spent on this project—and there was a considerable expenditure of both—as having been wasted.

It was, of course, disappointing that no practical synthesis of penicillin was forthcoming and that none of the simple synthetic compounds related to penicillin had therapeutic properties of any value, but it was important to know this in order that due weight might be given to improving the fermentation process for making penicillin and, in fact, a knowledge of the structure of penicillin helped to improve very materially the amount produced by the mould.

The complete results obtained in this collaborative investigation have now been published. During the 2½ years in which nearly 40 teams on both sides of the Atlantic collaborated, about 700 reports, officially classified as secret, were prepared and circulated. Although these served their immediate purpose of informing other workers of the progress made by any particular group, they were not suitable as a permanent record, and, when the time came for the results to be made public, it was decided to correlate the observations of all the groups and present them systematically and in detail in a separate monograph instead of in the scientific journals as originally proposed. The result is this well-produced volume, similar in page size and format to the *Journal of the American Chemical Society*. In each chapter, a description of the results obtained and the conclusions reached is given in ordinary type and is followed by the relevant experimental details in smaller type. The book is equipped with a good index.

The first chapter, containing a brief outline of the chemistry of penicillin, has already been published *verbatim* in *Nature and Science*. Of the other 28 chapters, the first three describe the results obtained prior to 1943 when collaboration commenced. The others deal with the chemistry of particular penicillin degradation products or of groups of substances related in one way or another to the penicillin molecule; with the infra-red absorption spectra of penicillin and related substances; with the application of X-ray analysis and other physical methods to the elucidation of chemical structure; with the biosynthesis of the penicillins; with chemical modifications of the penicillin molecule; with methods of assay; and with the various methods used to synthesise penicillin, most of which were so strikingly unsuccessful!

It is important that all concerned with chemical work in the pharmaceutical field should appreciate the uniqueness of this book. It is not a text-book in which existing knowledge is summarised and critically assessed, but a genuine source book containing information not available elsewhere. It has therefore the same status as the journal of a learned society and will

presumably be abstracted in the same way so that its contents may become known to those who have not ready access to the book itself.

"The Chemistry of Penicillin" covers a far wider field than its title implies. Chapters 21, 25 and 26, for example, contain probably most of what is known of the chemistry of oxazoles and oxazolones, thiazolidines and β -lactams, and subsequent work in these fields will doubtless contain many references to what may familiarly become known as *Chem. Pen.* Again, chapters 11 and 12 give an account of a new and extremely important method of determining the disposition of atoms within a complex molecule, that will doubtless be used increasingly in future work, whilst chapter 13 contains much fundamental data on the infra-red absorption spectra of penicillin, its degradation products, related compounds and simpler substances examined for purposes of comparison.

It is impossible in the space available to give more than this brief outline of the volume under review. It is a book that should be added to every scientific library of importance, especially as its price must put it beyond the reach of the individual chemist, and a book that every organic chemist interested in chemotherapy should browse through in order to familiarise himself with its contents; for it contains much unexpected information that may be of value in other fields.

F. A. ROBINSON.

ABSTRACTS (Continued from Page 633)

close agreement, with the possible and inexplicable exception of digoxin tablets. Results differed from those of the cat method by less than 10 per cent. in the majority of the samples. For the whole leaf products the maximum difference was found to be 19.9 per cent. Pigeons were less variable than cats for the assays reported and as a result fewer pigeons are required to meet the present U.S.P. requirement.

G. R. K.

Insulin, Potentiation of, by Sulphones. A. B. Macallum. (*Canad. J. Res.*, 1948, 26E, 232.) Sulphones in trace quantities combined with a diet rich in fresh vegetables were shown to produce in rabbits an increased sensitivity to insulin, both in the rate of fall of blood sugar levels and maintenance of hypoglycaemia. In order to relate the molar concentration of sulphone to the unit value of insulin, 1 ml. of a 0.01 M sulphone solution was used in conjunction with 1 unit of insulin. In the case of less soluble solutions more dilute solutions were used but the volume of the dose increased to keep the amount of sulphone in relation to the amount of insulin constant. The sulphone solutions were injected hypodermically into the side of the animal opposite the site of insulin administration in order to avoid formation of possible insulin-sulphone complexes. In the case of simple sulphones the potentiation did not appear until sulphamide was used, and the maximum effect in this group was attained with phthalyl tauramide. In the case of the sulphonamides the potency was least with sulphanilamide, but increased in the succeeding members of this series (sulphathiazole, sulphaguandine, sulphadiazine), the last, No. 307, a disulphone under experimental trial, being the most effective. The benzenesulphonic derivatives (ethyl benzenesulphonate, saccharin, benzenesulphonamide) were the most active of all the sulphones investigated. The sensitivity is not contingent on the presence of sulphone compounds, since it may persist for several

[Continued on page 637]

SCIENTIFIC MEETINGS

CHROMATOGRAPHY

Summary of a Lecture delivered by Professor A. H. Cook, D.Sc., Ph.D., at the Royal Institution

DR. COOK related how chromatography has helped in the isolation and purification of natural substances occurring in high dilution. This was especially so in recent years in the fields of hormones, vitamins, penicillin and vitamin B₁₂. Carotene, one of the first substances to be studied by the method, occurs at about 1 part in 1,000, lactoflavine is present at 1 part in 30,000 to 40,000 of culture medium and the anti-pernicious anæmia factor at about 1 in 80×10^6 . Another difficulty in the study of natural products lies in the accompanying impurities which are very often similar in physical and chemical properties and indistinguishable by ordinary chemical or physical methods. Chromatography frequently provides a solution to such problems. When there are great differences in the extent to which certain substances are adsorbed some of them may pass straight through the column, but they are not lost as they appear in the liquid at the end of the column in an orderly fashion. This is the principle of the liquid chromatogram. Sometimes a zone may contain more than one substance when it will be necessary to fractionate them by repeating the process in a number of columns. An American apparatus provides for up to 200 repetitions. Colourless compounds are detectable by viewing them in ultra-violet light, when many fluoresce, by the addition of a suitable dyestuff whose relative behaviour on the column is known or by the conversion of the substance to a coloured or fluorescent derivative. Chromatography has been the key to the chemistry of the carotenoids. It has also played an important part in micro-chemistry (detecting 0.01 g.) and in the examination of wines, foodstuffs and drugs.

The degree to which a substance is adsorbed is related to molecular structure. The carotenoids which are characterised by a varying number of OH groups and conjugated or isolated double bonds, are a good example of this.

Oxygen atoms, double bonds (conjugated more than isolated bonds) are associated with increased adsorption. Thus fucoxanthin containing 10 double bonds and from 4 to 6 OH groups is most strongly adsorbed whilst α -carotene is about the least. The stereoisomeric methyl bixins give chromatograms which vary with the different molecular shapes of these compounds. This method of identification is much more sure than that of melting-points.

Recently the method of mechanically separating the zones on a chromatogram has been replaced by one where the zones are eluted into a special container where the liquid is observed either polarographically or by continuous measurements of the refractive index. In this way Tiselius and Claesson have separated mixtures of lauric, palmitic and myristic acids. Ion exchange is a development of chromatography in which the ionic charge of the column and eluent plays an important part. It has been used to study the nucleotides and amino-acids. Substances which form salts in a medium of acid pH may be separated on a cationic column and those forming salts in one of alkaline pH on an anionic column.

Another recent development is the partition method in which the column is packed with silica gel containing water. The passage of the zones

down the column depends on their relative partition coefficients between water and the organic solvents. Adsorption by the silica is sometimes a problem which may be avoided by the use of strips or sheets of filter paper. This is a very convenient way of testing urine and has led to the detection of cysteine which was not formerly suspected in abnormal urine. The strip of filter paper is spotted with a drop of solution on a line drawn near one end. It is then suspended vertically in a glass cylinder so that the spotted end is immersed in a trough or organic solvent saturated with water at the top of the cylinder. The atmosphere is kept saturated with organic solvent and water vapour by placing the cylinder in a shallow dish of the mixture. The movement of the zone of substance in solution relative to the distance moved by the advancing front of liquid is measured. Various reagents are used to detect the zones of substances present. By using a sheet of filter paper a number of solutions may be examined at the same time and, under standardised conditions, it is possible to use maps to identify the different substances by their relative positions. Amino-acids both in the free and bound forms, gramicidin, penicillin, purines, sugars and anthocyanins have all been studied by this method. Vitamin B₁₂ was investigated by a modified method in which the chromatographic strip was laid across a seeded agar plate and the effect of the various zones on the bacterial growth was noted. Observations have been made on the products of photosynthesis in algae with the aid of radio-active particles. The cells were extracted with solvent and chromatograms prepared which were photographed on X-ray film. By comparison with chromatograms of known substances no less than 15 substances, hitherto unknown in photosynthesis, were detected. Partition chromatography has also shown the toxic factor in flour caused by treatment with nitrogen trichloride to be neither an amino-acid nor a protein. Because little change ensues in the nature of substances isolated by chromatography, it is indispensable in the investigation of natural products especially in such complex problems as the nature of bacterial toxins and the specificity of proteins. It has enabled us to speculate on the course of photosynthesis and the biogenesis of amino-acids.

ABSTRACTS (Continued from Page 635)

days after these compounds have been detoxicated or eliminated. The potentiating effect is due to the combined effect of the sulphone sensitisation and some element in fresh vegetables in the standard diet (fresh cabbage, lettuce, carrots, hay and oats).

S. L. W.

Testosterone, Long-Acting Preparation of. E. Carlinfanti, F. D'Alò and L. Cutillo (*Lancet*, 1949, 256, 479.) To a solution of 1 g. of crystalline testosterone in 10 ml. of alcohol (96 per cent.) is slowly added, with constant stirring, 20 ml. of an aqueous suspension of aluminium phosphate 7 mg. ml. The mixture is allowed to sediment and the supernatant fluid decanted off. The residue is made up to 40 ml. with saline solution. The preparation is stable and can be administered with a fine needle. Experiments were carried out on castrated guinea-pigs, one group receiving one injection of testosterone propionate in oil in a dose of 25 mg./100 g., and another group the same amount of pure testosterone adsorbed on to aluminium phosphate. It was found that one injection of the ester-in-oil preparation leads to a rapid rise and fall in the weight of the seminal vesicles, whereas the new preparation produces a greater and more continuous action, reaching a peak not earlier than 30 days after administration.

S. L. W.

NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Eulissin* is a synthetic curarising agent for use in anaesthesia and convulsive therapy. It is a sterile solution of decamethonium iodide (bistrimethylammonium decane iodide (C10) for intravenous injection. It is supplied in ampoules containing 5 mg. in 2.5 ml., in boxes of 6, 12 and 100 ampoules.

S. L. W.

Fel-evac* is a standardised fatty meal for use in cholecystography, replacing the unstandardised meal hitherto employed, usually consisting of eggs, with bacon or milk. The use of Fel-evac does not necessitate any departure from the usual procedure. A fat-free meal at 6 p.m. is followed an hour later by the opaque substance; only fluids are allowed until after the first radiograph at 9 or 10 a.m. the following morning. If a clear shadow is obtained, Fel-evac is then given and further radiographs taken at intervals of 15, 30 and 60 minutes. A dose of 1½ fl. oz. in a small glass of warm milk is usually satisfactory; in a normal gall-bladder this induces a good contraction within 30 minutes. It is supplied in 10 oz. and 20 oz. jars.

S. L. W.

Pyribenzamine* is N-benzyl-N-(α -pyridyl)-N'-dimethylethylenediamine monohydrochloride. It is a white, crystalline substance, which is stable, non-hygroscopic, and soluble in water. It is an anti-histamine, as little as 5 mg. protecting guinea-pigs against 50 mg. of histamine, or 100 times the lethal dose. It is indicated in the prevention and treatment of allergic conditions, such as allergic rhinitis, acute and chronic urticaria, allergic eczema, and drug reactions; it has also been employed in a number of pruritic conditions. Less encouraging results have been obtained in the treatment of bronchial asthma and migraine. It is administered in the form of tablets containing 0.05 g., the average adult dose being from 1 to 4 tablets daily. In young children dosage is determined on a body-weight basis, and in older children approximately one-half the adult dose is employed. Side-effects are usually mild, the commonest being drowsiness and gastro-intestinal disturbances; dryness of the mouth, vomiting and diarrhoea occur occasionally. Rare manifestations include insomnia, tachycardia, diplopia and urinary disturbances. It is issued in packages of 20, 100 and 500 tablets of 0.05 g., and as an elixir in bottles of 100 ml. containing 0.005 g. per ml.

S. L. W.

Visco-Pyelosil* is a 35 per cent. aqueous solution of diodone rendered viscous with a neutralised polymer of methacrylic acid and esters. Injected subcutaneously or intramuscularly, and introduced into the uterus and peritoneal cavity it causes no irritation. It must not be injected intravenously or intrathecally. It is employed as a radiological contrast medium in hysterosalpingography especially in the investigation of sterility. The miscibility with body fluids avoids globulation which can confuse definition or produce misleading appearances. If any enters the circulation it disperses rapidly and no complication from oil embolism can arise. Rapid dispersal and excretion also avoids risk of subsequent irritation due to unabsorbed contrast medium remaining in the cavities. The amount of contrast medium required usually does not exceed 10 ml. and all exposures should be completed within 10 minutes. It is issued in boxes containing 1 and 5 ampoules of 10 ml.

S. L. W.

NEW APPARATUS

A SIMPLE METHOD FOR PHASE-CONTRAST MICROSCOPY

PHASE contrast microscopy is particularly valuable in the examination of living cells with high powers and an ordinary microscope using an oil immersion lens may be easily adapted. This method was demonstrated by Dr.

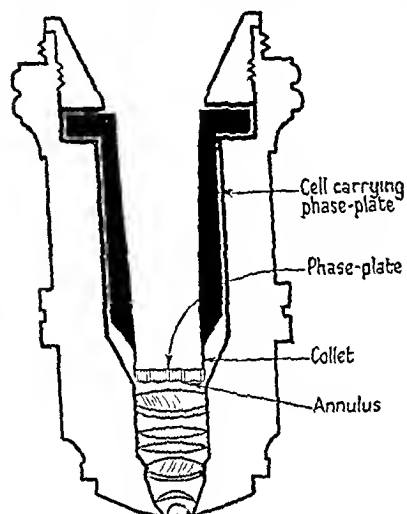


FIG 1.—Diagram of the microscope objective showing the method of fitting the phase-plate. (Reproduced by courtesy of the Quarterly Journal of Microscopical Science.)

J. R. Baker¹ and his associate workers of the Department of Zoology of Oxford University at the Royal Society's *Conversazione* on May 26, 1949. A phase-plate is prepared from a circle of glass, 1 mm. thick, of the same diameter as the back lens of the objective, and with the sides optically plane and exactly parallel to one another. This phase-plate is held in place behind the objective lens by means of a hollow brass cylinder which fits into the objective. The phase-plate is glued to the lower end of this cylinder (Fig. 1). The phase-plate is uniformly bloomed one side with magnesium fluoride to such a thickness as to give a retardation of a quarter-wave of apple green light, in comparison with light passing through the same thickness of air. (Deposition of magnesium fluoride is done by Messrs. R. and J. Beck.)

The plate, glued to the end of the cylinder, is accurately mounted in the centre of the revolving disc of a turntable used for mounting microscopical slides, and an annulus is dug out by scraping part of the bloom away with a chisel-pointed needle mounted on a specially constructed arm. The annulus should lie a little less than half-way from the centre of the phase-plate to its circumference.

In order to balance the direct light coming through the annulus with that of the diffracted light which passes through the rest of the phase-plate, carbon must be deposited on the annulus to reduce its transmission. This is done by passing the plate through a small xylene or benzene flame. The smoking should be sufficient to reduce light-transmission by about 30 per cent. The carbon is then removed from the phase-plate, except from the annulus itself. An illuminating annulus is next prepared. An annular space is cut away from a piece of black paper, which is then stuck on to a sheet of glass. The illuminating annulus is placed immediately in front of a 150 watt "Helios" enlarging lamp, which is the source of light. To set up the apparatus a square of fine ground glass is placed between the light source and the microscope mirror (Fig. 2). The microscope may now be used ordinarily. Some living cells are now placed on a slide and focused with a low-power objective. The condenser (preferably a high-power achromatic one) is then adjusted so that a pencil held against the ground glass is seen in focus with the cells. The low power objective is now replaced by the oil-immersion lens

NEW APPARATUS

carrying the phase-plate, and one of the cells is carefully focused. Then the slide is moved so that no cell is seen; the draw-tube is removed and a 3 in. objective is screwed into it at the bottom. The draw-tube is replaced and adjusted so that the phase-plate annulus is carefully focused. The sheet of

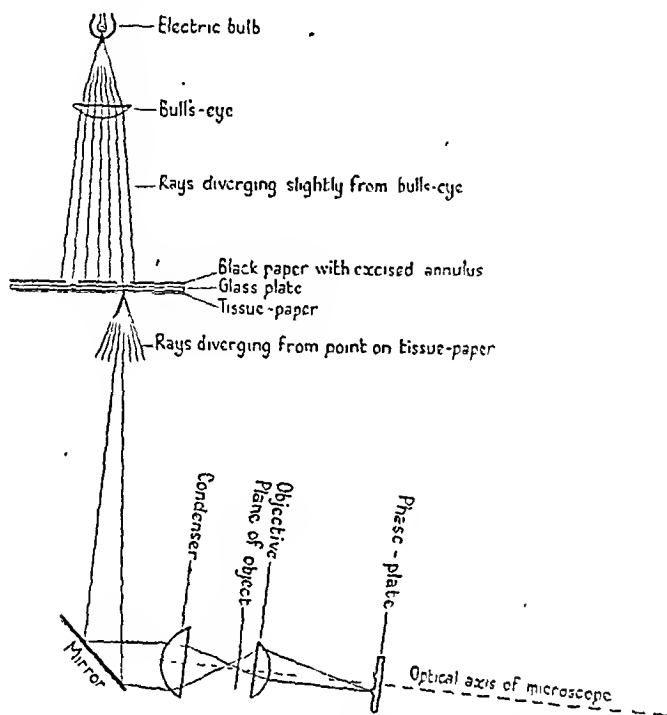


FIG. 2.—Optical diagram of the apparatus for Phase-contrast Microscopy. The system described in the text is an improved and simplified form. (Reproduced by courtesy of the Quarterly Journal of Microscopical Science.)

ground glass is next exchanged for the bright annulus, and the condenser is now lowered until the bright annulus is in focus at the same time as the phase-plate annulus. At this position, the image of the bright annulus thrown by the condenser lies in the plane that is conjugate to the plane of the phase-plate placed on the other side of the objective. To make the two annuli exactly coincide the mirror is adjusted and the bright annulus and lamp moved towards or away from the mirror, whilst corresponding movements of the condenser are made to keep the bright annulus in focus. The 3 in. objective is then removed and the object once more brought into the field of view. The microscope will show the cells in "positive" phase-contrast: the field will be bright, transparent objects of high refractive index will appear black or grey.

REFERENCES

1. Kempson, Thomas and Baker, *Quart. J. micr. Sci.*, 1948, 89, 351.

BRITISH PHARMACEUTICAL CONFERENCE BLACKPOOL, 1949

Chairman: NORMAN EVERS

CHAIRMAN'S ADDRESS

PHARMACEUTICAL RESEARCH

I PROPOSE, this afternoon, to talk on the theme of pharmaceutical research, its aims and scope and its relationship to research in medicine, chemistry and other sciences. This topic was discussed by Dr. T. E. Wallis in his address to the Conference in 1943, and I feel therefore, that some apology is necessary, but as I propose to approach the matter from a direction rather different from that of Dr. Wallis, who was mainly concerned with academic research, perhaps I may be forgiven for referring to the subject again. In fact, my address is in the nature of an addendum to Dr. Wallis's and will underline much of what he said.

In considering the subject of Pharmaceutical Research, I am immediately confronted by the difficulty of definition. What is Pharmacy and what is Research? The word "research" has become very much over-worked in these days. It seems to be applied to almost any type of enquiry. We hear of "listener research," "market research," "packaging research" and even "time-table research." Most people, I think, would hesitate to give the term "research" so wide a definition, but where is one to draw the line? Research cannot be defined according to the importance of the results achieved. The distinction between what is or is not research is something more intangible. Rather, it depends on the attitude of mind with which an enquiry is approached. A problem which appears at first sight to be simple and solvable by known methods and therefore not to be dignified by the name of research may turn out to involve a fundamental investigation into certain phenomena, which raises it to the plane of true research.

When I ask myself "What is pharmaceutical research?" I find it equally difficult to provide an answer. Pharmacy is not a science in itself. It is an art which makes use of many sciences. I would remind you of the words of Daniel Hanbury which were quoted by Dr. Wallis in his address and which I think are worth repetition. He said, "Our art, gentlemen, is ever progressive. All science is interesting to us since almost every scientific discovery may sooner or later, directly or indirectly, yield some results profitable to pharmacy."

It would be going too far to argue from this that all scientific research is pharmaceutical research. Here, I think, we must take into account the objective of the research, and say that pharmaceutical research is research carried out with a pharmaceutical objective, which is rather like saying that an archdeacon is one who performs archidiaconal functions.

What, then, is a pharmaceutical objective? I think that the best way to answer that question is to consider the different types of investigations

that can justifiably be claimed as pharmaceutical research. The following list is probably not complete, but, I hope, fairly comprehensive:

1. The synthesis of new organic compounds with the object of discovering new drugs, and the examination of known compounds for new pharmacological properties.
2. The isolation and purification of the active principles of naturally-occurring drugs, the elucidation of their structure and their synthesis.
3. Pharmacognostical research.
4. Research on methods of cultivation of vegetable drugs with the object of producing maximum potency.
5. Methods of preparation of drugs in a form suitable for administration, under which heading we may include the preservation and sterilisation of medicinal products.
6. The chemical and biological standardisation of drugs.

Let us consider these divisions in rather more detail.

The primary importance to medicine of organic chemical research needs no emphasis from me. The number of valuable synthetic drugs discovered in recent years provides sufficient evidence of this. The organic chemist must, of course, work hand-in-hand with the pharmacologist, who functions as a sort of compass and tells him whether he is moving in the right direction. Most research of this type is nowadays carried out by teams of workers, each approaching the subject with a special knowledge and skill. The organic chemist usually takes as his starting-point some compound, either natural or synthetic, of known pharmacological action, and by modification of its structure, produces new compounds which he hopes will have an improved or modified pharmacological action. Modification of structure in the direction of simplification has proved a fruitful source of new drugs. Such simplification may have the advantage of substantially reducing the cost of treatment. Examples of this are seen in stilbœstrol and related compounds which resemble in a much simplified form the structure of the natural œstrogens, and the new synthetic curarising compounds which are substitutes for the natural alkaloid, tubocurarine, are comparatively easily synthesised and are very much cheaper. Sometimes such researches lead the chemist far away from his starting point. The series of researches which started out to produce an improvement on the antimalarial drug, mepacrine, ended with paludrine, a compound of very different structure.

Research of this type sometimes involves the production of hundreds of compounds in the laboratory. The discovery of a successful new type of synthetic drug is followed by feverish activity in organic laboratories in the production of compounds of a similar structure in the hope of finding an improvement on the original. The number of sulphonamides which have been synthesised since the discovery of the value of sulphanilamide is legion, but the number of real value in medicine is probably less than a dozen. Sulphanilamide itself provides an example of a compound which has been known for many years before its valuable bacteriostatic properties were realised. This emphasises the importance of thorough pharmacological testing of all new compounds. Sometimes too, a varia-



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tion on a compound having a certain pharmacological action may prove to be valuable for a therapeutic effect of quite a different kind. On the other hand, valuable drugs are sometimes discovered which bear no relation in structure to those previously known. The analgesic, pethidine, is modelled on the structure of morphine, in amidone the relationship is scarcely recognisable. The new antihistaminic drugs are of a type not hitherto used in medicine and have a novel pharmacological action. The most remarkable development of recent years has been the discovery of drugs which act selectively on species of living organisms or upon one particular type of cell or enzyme. The antibiotics, anti-malarials, antrycide, etc., are examples of drugs acting selectively on certain organisms. Antihistamine, antithyroid, anticholinesterase and curarising agents are examples of selective poisoning of certain types of cells or enzymes. Increased knowledge of chemical constitution and its relation to pharmacological action, of the causes of disease and of the nutritional requirements of organisms makes the task of the organic chemist less subject to chance than it used to be, but we are still a long way from being able to design a new drug like a machine on a drawing-board. The element of luck has not yet been entirely eliminated, but as Pasteur wisely said, "In the fields of observation chance favours only the mind which is prepared." It may have been a lucky chance that led Sir Alexander Fleming to the discovery of penicillin, but unless his mind had been prepared by years of research and thought on such matters, who knows that he would have appreciated its significance?

It cannot be too strongly emphasised that the greatest possible care and the most thorough and exhaustive trials are necessary before placing a new drug on the market. Time and again a drug, which has been thought from preliminary tests to have an irreproachable character and has been launched with a flourish of trumpets, has proved to possess undesirable and even dangerous qualities.

The second type of research to which I have referred deals with the isolation and purification of natural drugs, the determination of their structure and synthesis. Recent discoveries have shown that Nature still has something up her sleeve. A few years ago it might have been thought that it was unlikely that any new natural drugs of value would be discovered and that the future lay with synthetic organic chemistry. Then came penicillin to show us an entirely new type of drug from an entirely new source—a compound with a new sort of structure, and one, moreover, that has not yet been synthesised except in minute quantity in spite of the efforts of some of the finest organic chemists in the world—a blow to the pride of those organic chemists who might have been inclined to think that anything can be synthesised once its structure is known—except, of course, cane sugar. Then, as if to heap coals of fire on the head of the organic chemist, Nature produces chloramphenicol or chloromycetin, a naturally occurring antibiotic with a comparatively simple structure, containing, above all things, a nitro-group and two

chlorine atoms, looking for all the world like a typical product of the organic laboratory, yet it is not among the hundreds of thousands of compounds which have been synthesised. Truly it behoves those who pry into the secrets of nature to preserve a spirit of humility.

Another natural product which promises to be one of the important drugs for the relief of human suffering is known as "Compound E," obtained from the suprarenal gland. The minute amount present in the gland precludes its extraction from natural sources in quantity, and all hopes are centred on a successful synthesis. The known synthesis is long and difficult, even if supplies of raw material from *Strophanthus sarmentosus* are forthcoming in sufficient quantity. The position is a challenge to the organic chemist, and those who solve it will, indeed, have deserved well of mankind.

I do not wish to spend more time on the contributions of organic chemistry to pharmaceutical research. Fundamental as these are, we must in these days regard organic chemistry as a highly specialised art and the organic chemist as a species apart. The pharmaceutical student who intends to become an organic chemist must realise that, henceforth, his whole mind will be steeped in organic chemistry and perhaps in one small section of this vast subject.

I cannot speak with any authority on the third section of pharmaceutical research on my list—pharmacognosy. Dr. Wallis has already done so, nor can I say anything on the fourth item—the cultivation of drugs, so I will pass on to the fifth, the section of pharmaceutical research which most properly merits the name, the preparation of drugs in a form suitable for administration. The introduction of a new drug into medicine entails four steps, (a) the chemical production by synthesis or other means, (b) the pharmacological investigation, (c) the pharmaceutical investigation and (d) the clinical trial. The chemical research should supply information on the physico-chemical properties of the drug, the solubility etc., and on its stability to heat, moisture and oxidation. The pharmacological research should give data on the best means of administration and the probable dose required, and some information about the method and rate of absorption and excretion, whether the effect is transient or prolonged. The clinician can advise from such data on the type of pharmaceutical preparation which is most suitable for administration, whether dosage should be frequent, or whether the action should be prolonged by some such means as the use of an oily medium for the injection. Nevertheless the pharmacist is by no means the least important link in the chain. Failure to provide a satisfactory pharmaceutical preparation may bring a new drug into discredit. The pharmacist out of his experience can and should give valuable assistance to the clinician as to the best method of administration of a new drug.

If an injection is required, a number of questions must be considered, such as—

(a) Is the drug sufficiently soluble and stable in water to make an aqueous injection possible? If not, can any other solvent be used or can

any addition be made to increase the solubility? Would any other form of the drug, such as another salt, be more suitable for injection? If these questions cannot be answered satisfactorily, would a dry ampoule be a suitable method? (b) Is the addition of sodium chloride or other material necessary to make the injection isotonic? If so, how does it affect the solubility or stability of the drug? (c) Is the natural pH of the drug suitable for injection, and does it ensure the maximum stability or must the pH be stabilised by the addition of a buffer? (d) What is the most suitable preservative, if one is required, and is it likely to react with the drug or affect its stability? (e) What is the most suitable method of sterilisation? Does sterilisation by heat cause any decomposition of the drug or production of toxicity? If heat is unsuitable, does sterilisation by filtration cause any loss of potency? (f) Should the injection be protected from oxidation by filling the ampoule with nitrogen? (g) What is the stability of the injection as finally formulated under ordinary conditions, under tropical conditions or under abnormally cold conditions? (h) If the injection is to be in a rubber-capped container, will contact with rubber affect the injection in any way? (i) Should the injection be protected from light by the use of amber containers?

If the required form is an oily solution or suspension, other problems arise such as— (a) What is a suitable composition for the oily base? (b) If a suspension is required, what is the most suitable particle size of the drug? (c) Does the drug remain easily dispersible in the base? (d) Is the viscosity suitable for drawing into a syringe?

If the new drug is likely to be used in combination with some other drug a study of any possible interaction between the two must be made.

The investigation of all these problems may involve a considerable amount of work. A large number of different formulae may have to be tried and each one checked either by analysis or by pharmacological tests or both. If the drug is a new one, analytical methods may have to be devised in order to detect decomposition. If the drug is administered orally, the problems are not usually so complex. The pharmacist must first consider whether a tablet is a suitable medium of administration or if not, whether a capsule would be a better form. If a tablet is chosen, the compatibility of the drug with the usual diluents or lubricants must be considered, and the amount and the type of diluent necessary to give effective disintegration of the tablet, the possible effect of the granulation process on the drug, and the stability of the drug in tablet form.

New drugs may sometimes call for new methods of administration. I should like, if I may, to take an instance from my own experience of what I regard as an example of pharmaceutical research, which called for all the resources of the pharmacist as distinct from the chemist, and in my opinion, was a type of problem which the pharmacist can tackle better than anyone else. I refer to the production of a chewing-gum containing penicillin. Here was a problem which was quite new. It was not a question of adding penicillin to the ordinary chewing-gum base, because the ordinary chewing-gum base contains water, and penicillin

would not last in it for more than a few days. A completely new type of water-free base had to be produced, and the penicillin had to be incorporated in such a way that it was liberated over several hours of chewing. The chief asset of the pharmacist, as I said in my address last year, is his knowledge of the properties of materials. It is said that the organic chemist with the aid of some coloured balls and bits of wire can design a new detergent and confidently prophesy its properties before the compound is made. A chewing-gum cannot be designed in this way, since so little is known of the effects of constituents on the rheological properties of solids. Many experiments had to be made before the right consistency was reached and the desired slow release of penicillin was attained, involving many lengthy chewings by rather unwilling human guinea-pigs and the taking of many samples of their saliva for penicillin assay. Not only must the desired consistency be attained, it must be retained under a variety of storage conditions, for the margin between chewing-gum which is too soft and sticks to the teeth, and chewing-gum which breaks up when chewed is a very narrow one. There is also the problem of flavouring to be considered; this, too, must be liberated slowly and must not react with the penicillin. Problems such as this can only be solved when the accumulated knowledge and experience of the trained pharmacist is brought to bear upon them.

Drugs which are used by external application provide some of the most interesting problems for the pharmacist. Many of the physical problems which arise in the formulation of such products were mentioned in my address last year, and I do not propose to repeat them now.

Much of the research in industrial laboratories is concerned with methods for large-scale production, not merely of new drugs, but of pharmaceutical preparations. Advice must be given to the production department on the most suitable type of plant and for this purpose batches of a size comparable with a production batch must be carried through. Transfer from the laboratory bench scale to the manufacturing scale is rarely achieved without difficulties, even though an intermediate or "pilot" stage is interposed. The closest co-operation between the research and production staff is essential to ensure success.

But research is not confined to new drugs or to new methods of presentation of drugs. There is a continual stream of problems arising from existing preparations. New ingredients are introduced and reformulation is required to produce a new and improved product. Improved types of plant may require a modification of a production process. Contact with the production department may lead the research worker to suggest an improvement in the production process which will give greater efficiency or reduce the cost. Existing formulae may be found to be unsatisfactory when subjected to certain conditions, or some slight alteration in the composition of one of the constituents may have caused trouble in the finished product. The question of stability provides more problems and more headaches for the pharmaceutical research laboratory than any other. The hospital and retail pharmacist is not so much concerned with this problem, but to the manufacturer it is all important, especially if

goods are to be exported. All sorts of conditions of temperature and humidity must be provided for, and in many cases it may be necessary to use a different formula for tropical countries. Suggestions from clinicians for improvements in the administration of drugs or for new combinations of drugs are a fruitful source of investigation for the pharmaceutical research worker. It is a curious fact, that, no sooner is a new drug introduced, than suggestions flow in for combining it with other drugs. The belief of some clinicians in synergism seems to be unbounded.

The proper organisation of research means a ready access to the published literature on the subject. Much of the research worker's time can be saved by an efficient library service and indexing system. The subject of patents is the bugbear of the industrial research worker. A knowledge of existing patents is essential before undertaking a piece of research. This is not the place to discuss the merits or demerits of patents for medicinal products, but certainly the number of patents granted in this field is increasing and rendering the task of the research pharmacist more difficult. One result of this is the multiplication of *new drugs* to the confusion of the medical man and the pharmacist. The success of a new drug induces rival manufacturers to produce a similar compound with a modification in structure which avoids the original patent, but may or may not possess any therapeutic advantage. The number of antihistamine drugs which have been put on the market in the United States has forced the Council on Pharmacy and Chemistry of the American Medical Association to refuse to accept new products unless they show a very marked superiority over the old.

Familiarity with recent advances in the pharmaceutical field is a necessary equipment for the research worker, but advances in other industries may provide the key to the solution of many a pharmaceutical problem. The greater part of pharmaceutical research is carried out in the laboratories of industrial firms, though much is done in the Universities and Schools and by hospital pharmacists. The days when the retail pharmacist can spare much time for research work have unfortunately passed; the time when men like Farr and Wright could spend laborious hours on the determination of alkaloids in drugs is no more. Most modern research requires expensive equipment which is not found in the pharmacy. Nevertheless, the retail pharmacist of an inquiring mind must meet many problems which he could solve for himself with some expenditure of time and ingenuity. Doubtless much work of this type is done, but with a few exceptions such efforts remain "unhonoured and unsung." It is to be hoped that the National Health Service will not still further discourage such efforts. Pharmacy must not become mass produced. The spirit of inquiry which is, or should be, engendered in pharmacists by their scientific training should not be stifled when they engage in practice.

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of pharmaceutical research by a committee such as was envisaged by the Executive Committee of this Conference in their report to the Council of the Pharmaceutical Society. Such a body, composed of men of distinction in pharmacy and the allied sciences, could do much to influence the direction of research towards the most pressing problems and to ensure co-operation between those interested in the same problem from different points of view. There seems to be a need for increasing the amount of research work which is carried out on behalf of the British Pharmacopœia and the British Pharmaceutical Codex. Pharmaceutical research must always be the hand-maiden of medical research, but this does not mean that it must be entirely dependent upon it. In fact, the introduction of new or improved pharmaceutical products can and does influence methods of administration. A method of prolonging the action of a drug by altering the form of presentation may so reduce the number of doses required that treatment is much less irksome to the patient.

I have not left much time to deal with the sixth item on my list—research on the chemical and biological standardisation of drugs. The discovery of a new drug is dependent on some method of testing its efficacy. The isolation of an active principle from a vegetable or animal source is dependent on some method of assaying its potency. New drugs require new methods of assay, whether chemical or biological. New methods of manufacture may introduce new impurities. Methods of testing are continually being improved. In parallel, therefore, with research proper must go research on methods of control. Such work can be just as fascinating as other types of research, though the prizes may not be so valuable.

The ideal type of research worker is rare and it is unlikely that all members of a research team will have the true research mentality. The laboratory might not be a very pleasant place to work in if they had. The plodder is needed as well as his more brilliant colleague. There is a general and laudable desire among students of science nowadays to become research workers. The first-class research worker must be a person of ideas, but ideas alone are not enough. He must have the capacity of sorting out the ideas which are practicable and those which are not. He must have the capacity for perseverance and concentration which are necessary to carry an idea into effect. He must be an enthusiast and be able to inspire enthusiasm in others, but his enthusiasm must not carry him so far that he ignores unpleasant facts. He must be prepared to meet continued failure and rise with head "bloody but unbowed." Above all, he must know when to stop. He must be prepared to accept much of his reward in satisfaction with work well done. He should be able to say, as was said by an old chemist a few hundred years ago, "The chymists are a strange class of mortals impelled by an almost insane impulse to seek their pleasure among smoke and vapour, soot and flame, poisons and poverty; yet among all these evils, I seem to live so sweetly. that, may I die, I would not change places with the Persian King."

RESEARCH PAPERS

THE ISOLATION AND IDENTIFICATION OF BEETLE FRAGMENTS FROM POWDERED VEGETABLE DRUGS

By COLIN MELVILLE

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INTRODUCTION

VEGETABLE drugs are required by the British Pharmacopœia 1948 to be free from insects and other animal matter and it follows from this statement that such matter is not to be tolerated under the term "foreign organic matter." Most vegetable drugs whether whole or powdered are very prone to attack by insect pests especially when carelessly stored. The presence of whole insects is readily detectable by examining the material directly or by sieving. If infested material has been subsequently powdered, any eggs present are usually killed and unless reinfestation occurs no whole insects will be found. Detection must then be based on whatever insect fragments are present and since most drug pests are very small creatures, the weight of the more common beetles being of the order of 1 mg. and that of the identifiable fragments much less, even high infestations may be overlooked by direct microscopical examination. Some method of concentrating the insect fragments must therefore be adopted. The identification of fragments as being of insect origin is not however sufficient evidence that the material was infested. Certain drugs, particularly herbs such as hyoscyamus, not infrequently contain small insects which were associated with the growing plant and were not removed during the preparation for the market. Large insects such as the cockroach may also occasionally find their way into drugs and unless their identity was realised, the powdered material might be reported as highly infested. Thus a study of the diagnostic microscopical characters of drug pests appears desirable. In the present paper, methods for the isolation of insect fragments are considered, and the diagnostic microscopical characters of some of the more common beetle pests of drugs described.

THE ISOLATION OF INSECT FRAGMENTS

For purposes of identification, the soft parts of insects may be ignored and only the exoskeleton need be considered. This consists of chitin impregnated with sclerotin and other substances, and is resistant to many chemical reagents, including boiling dilute mineral acids. Boiling caustic alkalies tend to remove the colouring and hardening materials without producing other visible changes. The exoskeleton is also water repellant but is readily wetted by petroleum and similar non-polar liquids.

Thus two methods of isolating insect fragments from powdered drugs are available; floating off with a non-polar liquid from an aqueous suspension, or solution of the vegetable material. Flotation methods are used extensively for the examination of foodstuffs and are particularly

suitable for starchy materials containing little cellulosic matter. In the author's experience, they have not afforded the complete separation of insect fragments when applied to powdered drugs. A method involving solution of the cellulosic material of infected drugs was devised by Greenish¹, but does not appear to have been generally adopted. It consists of boiling the defatted powder in 5 per cent. hydrochloric acid, macerating the washed residue for 18 to 48 hours in a mixture containing about 12.5 per cent. of sulphuric acid and 20 per cent. of chromic acid and separating the insect fragments by centrifugation. This method is not entirely satisfactory because solution of the vegetable material is not always complete and because of the time involved. The use of acetolytic methods appeared to the author to be more suitable and it was decided to investigate their practicability. Preliminary experiments with the usual laboratory methods of acetylation were made on absorbent cotton and on tow (lignocellulose). These materials dissolved most readily and completely in acetic anhydride containing 10 per cent. of concentrated sulphuric acid. With vegetable drugs however, solution was not always complete and an amorphous sludge sometimes remained. This was thought to be due to the non-cellulosic constituents since the crude fibre from these drugs was found to dissolve completely. After various trials the following method was evolved.

Method.—Boil about 5 g. of the powdered drug in a flask with 100 ml. of 10 per cent. w/w nitric acid in water for about 1 minute with frequent shaking. Filter through a No. 3 sintered glass filter, using suction, and wash the residue with hot water. Return the residue to the flask and boil for about 1 minute with 100 ml. of 2.5 per cent. sodium hydroxide solution, filter through the original filter and wash the residue with hot water. Remove the excess of water by suction and the last traces by passing a few ml. of glacial acetic acid through the filter. Transfer the residue as completely as possible to a flask of about 50 ml. capacity, and wash the remaining fragments into it with 10 ml. of acetic anhydride. Add a mixture of 10 ml. of acetic anhydride with 2 ml. of concentrated sulphuric acid, mix well and heat on a boiling water-bath until the crude fibre has dissolved. Solution is usually complete after about 10 to 15 minutes during which the liquid becomes dark reddish brown. Separate the residue by centrifugation, pour off the supernatant liquid, replace with glacial acetic acid and recentrifuge. After pouring off the glacial acetic acid, the residue of insect fragments may be mounted in any desired mountant for microscopical examination. Alternatively, the acetic anhydride can be hydrolysed before centrifugation by gradually adding the contents of the flask to about 10 ml. of water. Where infestation is heavy and the finest fragments are not required, the contents of the flask may be passed through the sintered glass filter and the residue washed with glacial acetic acid followed by water. It can then be removed by adding a few drops of water or mounting fluid to the filter and gently brushing with a small stiff brush. Passage of air the reverse way through the filter by connecting the side-arm of the receiving flask to a water tap facilitates the removal. If the drug contains much oil or fat it is pre-

ferable to remove most of it by maceration for a short time with one or two changes of light petroleum or similar solvent before preparing the crude fibre.

IDENTIFICATION OF THE FRAGMENTS

The beetles most frequently infesting stored vegetable drugs² are *Stegobium paniceum* L. (the drug-room beetle) and *Ptinus tectus** Boic. (the brown spider beetle). Less common but of frequent occurrence are *Niptus hololeucus* Fald. (the golden spider-beetle) and *Calandra granaria* L. (the grain weevil). *Lyctus brunneus* Steph., one of the powder post beetles, although primarily a pest of timber has been reported on vegetable drugs e.g. liquorice³. In these laboratories it has been found on Butea seeds and was recently introduced on Shensi rhubarb on which it appears to thrive. The following account is confined to these five species with the addition of a reference to the common cockroach *Blatta orientalis* L.

Materials.—A sample of Jamaica ginger was examined for freedom from infestation and then coarsely chopped. For each species 10 beetles were mixed with about 30 g. of the material and powdered in a steel laboratory end-runner mill until fine enough to pass through a No. 60 sieve. About 5 g. quantities were treated by the method described above and the residue mounted in cedar-wood oil for microscopical examination. Further mixtures with other drugs were subsequently prepared and the residue after removal of the vegetable material also examined. It consisted of fragments from the added beetles together with the acid-insoluble ash if any. With some drugs, especially leaves or herbs, the residue often contained pollen, some foreign to the particular drug, also various fungal spores and hyphæ.

The following descriptions were made from these fragments after reference to the whole insects cleared by boiling in 2.5 per cent. sodium hydroxide solution. With a few exceptions indicated by the absence of a surrounding line, the sketches were also made from the fragments. Drawing was done with the aid of a camera lucida at an original magnification of $\times 500$, or for mandibles and antennal joints $\times 200$.

Microscopical Characters. General.—The fragments consist of the cuticle of the beetle, the soft parts having been dissolved. Fragments from the body are irregular in outline, yellowish-brown and translucent. The larger joints of the appendages are also broken but the smaller ones such as those of the antennæ and tarsi are often intact. In life, the beetles are clothed with hairs all but a few of which become detached during the powdering process, but leave scars to mark their position and frequency. The detached hairs are usually too finely broken to be recognisable although occasional ones are found intact. The hairs of the species described are of two general types, bristle-like articulated setæ, and smaller and relatively more numerous clothing hairs. On the fragments, the positions of the former are marked by the setal scars which consist of a circular puncture enclosed within a concentric ring whereas the clothing

* Wallis mentions *P. brunneus* Dft. but *P. tectus* appears the more common of the two species.

hairs leave minute usually simple scars referred to subsequently as micro-punctures. The fragments proving of most value for identifying the beetles were derived from the elytra, the prothorax and the head, those from other segments being less characteristic. Elytral fragments are recognised by the presence of coarse punctures arranged in rows—the stria punctures, which usually have a densely coloured rim. They are surrounded by an oval or irregular area slightly darker than the general colour and apparently due to a local thickening or ingrowth of the cuticle. These areas may be absent from some fragments if the cuticle has laminated. In the majority of cases, the rows of stria punctures are parallel except on fragments from the ends of the elytra. In certain species, coarse punctures resembling the stria punctures occur elsewhere than on the elytra. These can be recognised either by their irregular arrangement or if they are in rows, by their closeness to the rim of the segment which is usually also present.

The outer surface of the fragments may be smooth or raised into tubercles or ridges. The latter often enclose polygonal areas which probably correspond with the original cells of the epidermis. Mandibles are rarely found unbroken but the thick biting edge is often intact in which case the number and arrangement of the teeth is a useful character. The shape and size of the terminal joints of the antennæ is also of value, while their number together with that of the mandibles gives an indication of the number of beetles originally present.

STEGOBIUM PENICEUM L.

Elytra: stria punctures 10 to 50 μ apart in parallel rows, slit-like about 20 to 30 μ long, with on each side one or rarely two rounded tubercles, each tubercle associated with a micro-puncture; the whole surrounded by an oval area about 30 to 50 μ long; intervals between the rows 100 to 150 μ broad, with setal scars about 10 to 15 μ diameter, mainly in a single median row and separated by about 20 to 70 μ ; over the entire surface, numerous scattered micro-punctures separated by up to about 20 μ . (Fig. 1.C.)

Prothorax: tuberculate; tubercles prominent, rounded conical, furrowed and irregularly dentate at the base, about 10 to 20 μ diameter, contiguous or separated by up to about 50 μ , each closely associated with a setal scar; intervals micropunctate like the elytra. (Fig. 1.E.)

Head: dorsal surface similar to the prothorax but with less prominent tubercles; ventral surface ridged, ridges curved to semicircular, distinct or confluent forming crenate rows, each ridge overhanging a shallow depression containing a hair or its scar. (Fig. 1.A.)

Antenna: terminal joint ellipsoidal about 250 μ by 70 μ , constricted at the base, hairy. (Fig. 1.F.)

Mandible: biting edge about 150 μ long, tridentate, the teeth diminishing in size from apex to base (Fig. 1.B); in the larva, the subterminal teeth prominent and connected by a cutting edge. (Fig. 1.G.)

Hairs: setæ; cylindrical, about 100 to 150 μ long, acuminate, moderately thick walled, straight or slightly curved; clothing hairs similar but smaller, about 30 to 50 μ long. (Fig. 1.D.)

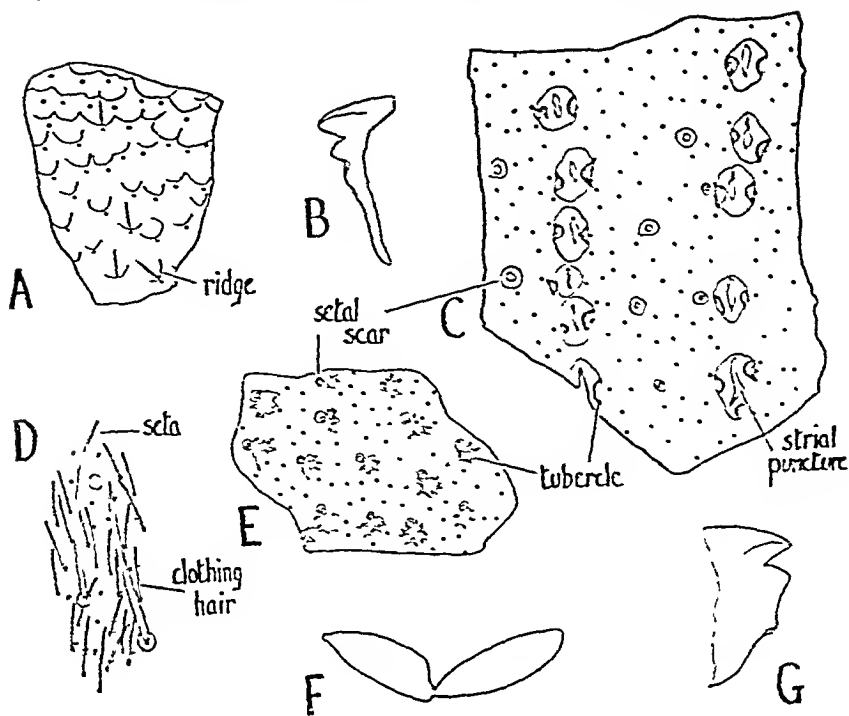


Fig. 1. *Stegobium paniceum* L.

A. Head, fragment from ventral surface showing ridges and an occasional hair, $\times 200$. B. Biting edge of a mandible. G. The same from a larva, $\times 80$. C. Elytron, fragment from the middle region showing two rows of strial punctures, $\times 200$. D. Hairs from elytron of a whole insect, $\times 200$. E. Prothorax, fragment showing tubercles with associated setal scars, $\times 200$. F. Antenna, terminal and subterminal joints, hairs not shown, $\times 80$.

PTINUS TECTUS BOIE.

Elytra: strial punctures up to about 30μ apart in parallel rows, oblong to elliptical about 25 to 50μ long with a thickened rim and surrounded by an oval or rounded area about 60 by 55μ ; intervals between the rows 80 to 100μ broad, with a single median row of setal scars about 5 to 7μ diameter and separated by 40 to 60μ ; over the entire surface, numerous scattered micropunctures separated by up to about 20μ . (Fig. 2.A.)

Prothorax: central region of the pronotum coarsely punctate, punctures irregularly arranged, deep, circular to irregularly oblong about 40 to 80μ long, resembling the strial punctures of the elytra but sometimes lacking the surrounding area; intervals with scattered setal scars and micropunctate like the elytra, Fig. (2.D): remaining regions locally thickened, thickened areas isolated, more or less circular and surrounding a setal scar, or confluent, irregular and enclosing up to about 5 setal scars; surface over the areas micropunctate, intervals impunctate. (Fig. 2.C.)

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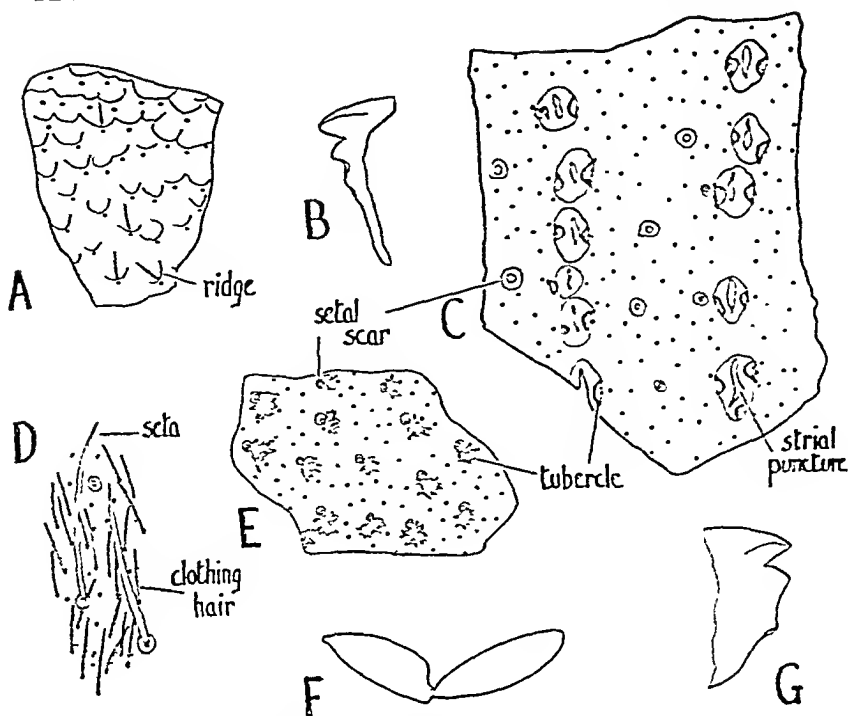


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the elytra. (Fig. 2.B.) Similar fragments from the thoracic and abdominal segments also occur.

Antenna: terminal joint ellipsoidal, about 270 by 100 μ and 30 to 40 μ in diameter at the base, hairy. (Fig. 2.F.)

Mandible: biting edge about 120 μ long with two teeth, apical tooth acute, basal tooth subacute. (Fig. 2.G.)

Hairs: setae; resembling those of *S. paniceum* but rather longer and

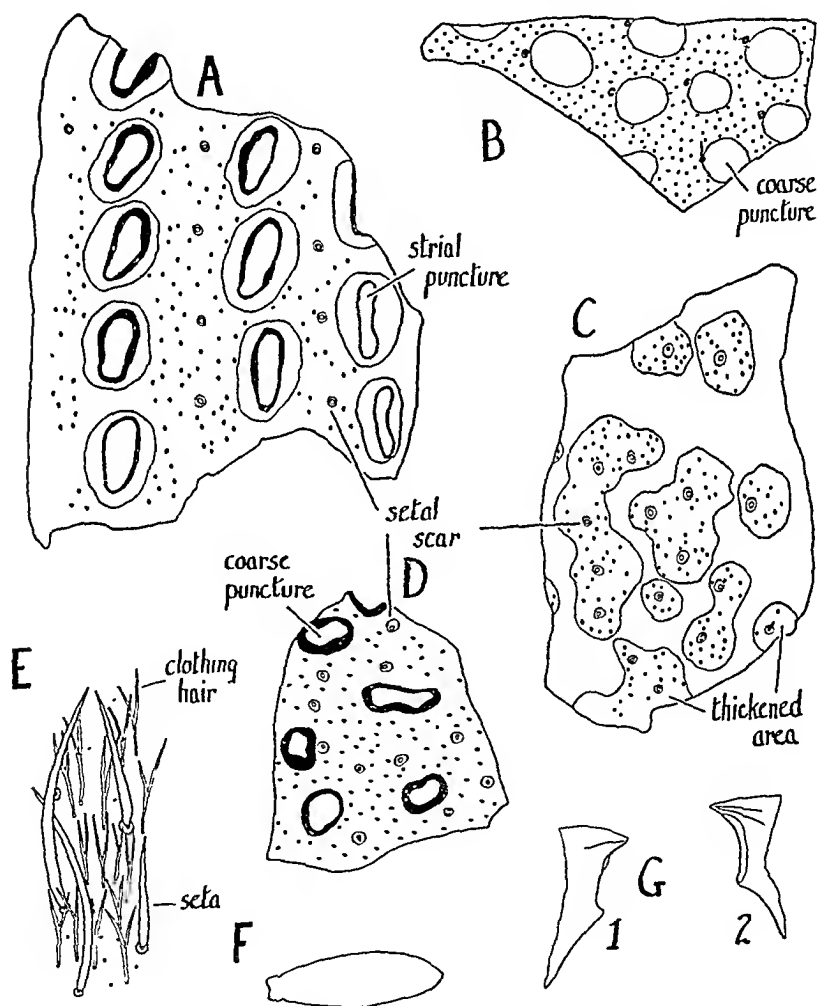


Fig. 2. *Ptinus tectus* Boie.

A. Elytron, fragment from middle region. B. Head, fragment showing coarse shallow punctures with associated setal scars. C. and D. Prothorax; C, fragment from near the head showing isolated locally thickened areas; D, from central region of the pronotum showing coarse deep punctures. E. Hairs from elytron of a whole insect. A to E, $\times 200$. F. Terminal joint of antenna, hairs not shown, $\times 80$. G. Biting edge of mandibles: 1. larval; 2. adult, $\times 80$.

wider; clothing hairs Y-shaped, cylindrical with acuminate arms, about 40 to 60 μ long. (Fig. 2.E.)

NIPTUS HOLOLEUCUS FALD.

Elytra: stria punctures about 100 μ apart in parallel rows, circular about 5 to 10 μ diameter with a deeply pigmented rim about 20 μ diameter, each surrounded by a rounded to oblong concentrically striated area about 120 by 80 μ having an indented margin and sometimes exhibiting a number of fissures radiating from the puncture; co-linear with the stria punctures, a row of setal scars, one associated with each puncture and separated from it by 20 to 40 μ *; intervals between the rows about 200 μ broad, with setal scars about 5 to 10 μ diameter mainly in a single median row and separated by about 50 to 100 μ ; over the entire surface numerous scattered micropunctures separated by up to about 20 μ . (Fig. 3.A.)

Prothorax: over the greater part, scattered setal scars and micropunctures similar to those of the elytra; near the margin one or sometimes two rows of coarse punctures separated by about 30 to 70 μ , punctures deep, circular to oblong about 20 to 40 μ long with a thickened rim and sometimes an enclosing circular to oval area about 40 to 80 μ long. (Fig. 3.C.)

Head: over the greater part, scattered setal scars and micropunctures; near the margin tuberculate, tubercles contiguous or separated by up to about 20 μ , conical, each subtending a short hair; extreme margin reticulately ridged, the ridges enclosing elongated polygonal areas about 15 to 60 μ long and 15 μ wide. (Fig. 3.F.)

Other regions; meso- and metasternites, some joints of the appendages: sparsely micropunctate and reticulately ridged, the enclosed areas more or less regular penta- or hexagonal, about 10 to 15 μ long and 5 to 10 μ wide. (Fig. 3.G.)

Antennae: terminal joint ellipsoidal to subcylindrical, about 400 by 100 μ and 50 μ in diameter at the base, hairy. (Fig. 3.B.)

Mandibles: biting edge about 200 μ long with two teeth and resembling that of *P. tectus*. (Fig. 3.E.)

Hairs: setae, cylindrical to narrow fusiform, about 100 to 150 μ long, acute or bifurcate at the tip, relatively thick walled; clothing hairs, flattened scale-like about 40 to 60 μ long by 10 to 20 μ wide, cleft into three or occasionally four long fine points. (Fig. 3.D.)

CALANDRA GRANARIA L.

Elytra: surface corrugate with intervals of 40 to 100 μ : in the furrows, one or rarely two rows of circular punctures about 5 to 10 μ diameter each situated at the bottom of a circular or oval saucer-shaped depression about 15 to 30 μ long and separated by about 40 to 70 μ , each depression also containing a circular puncture about 3 to 5 μ diameter or an occasional hair: intervals, reticulately ridged the ridges enclosing regular or somewhat elongated hexagonal areas about 10 to 30 μ long and 10 μ wide, and also exhibiting an occasional punctured depression like those of the furrows. (Fig. 4.B.)

* Hinton* states that the setae arise from the stria punctures.

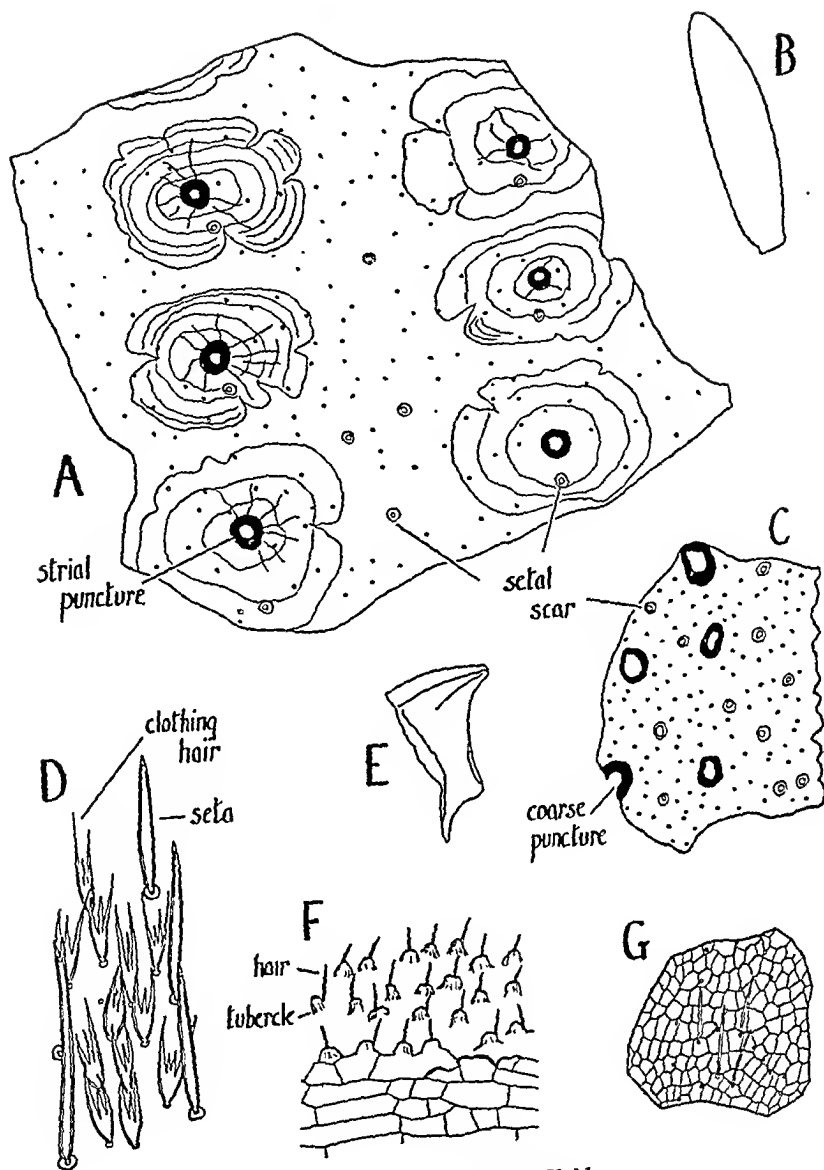


Fig. 3. *Niptus hololeucus* Fald.

A. Elytron, fragment from middle region showing strial punctures with surrounding areas and associated setal scars, $\times 200$. B. Terminal joint of antenna, hairs not shown, $\times 80$. C. Prothorax, fragment including the margin, showing coarse punctures, $\times 200$. D. Hairs from elytron of a whole insect, $\times 200$. E. Biting edge of a mandible, $\times 80$. F. Head, fragment from near the margin showing tubercles with associated hairs and reticulate ridging, $\times 200$. G. Fragment from mesosternite showing reticulate ridging and occasional hairs, $\times 200$.

Prothorax, head and other regions: sculpture like that of the elytra, punctured depressions often larger, up to 60μ diameter, scattered irregularly or arranged in more or less regular rows, and separated by about

BEETLE FRAGMENTS FROM POWDERED VEGETABLE DRUGS

15 to 160 μ , reticulations more regularly hexagonal than on the elytra, about 5 to 10 μ long. (Fig. 4.A.)

Antennae: terminal joint obovate, up to about 300 by 150 μ , diameter at the base about 50 μ , apical fourth with numerous very small hairs, remainder almost glabrous. (Fig. 4.D.)

Mandibles: biting edge curved, about 100 μ long with 3 or 4 closely arranged serrate teeth. (Fig. 4.C.)

Hairs: arising from the punctured depressions, about 25 to 50 μ long, somewhat flattened, multifid, with 5 to 10 linear truncate segments often unequal in length; the base sometimes elevated on a bun-shaped tubercle.

LYCTUS BRUNNEUS STEPH.

Elytra: stria punctures 10 to 25 μ apart in parallel rows, narrow elliptical to slit-like about 5 to 15 μ long, with a thickened rim and enclosed in an oval or irregularly oblong area about 15 to 30 μ long: in-

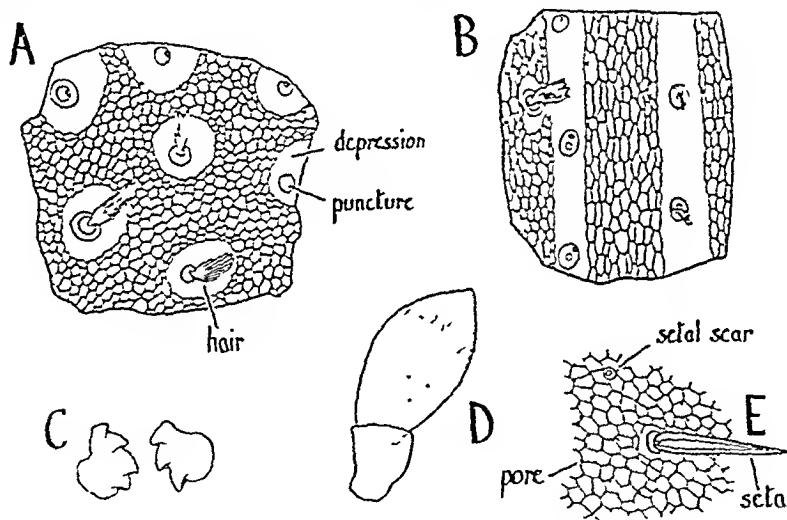


Fig. 4. *Calandra granaria* L.

A Prothorax, fragment showing hairs arising from the saucer-shaped depressions, with reticulately ridged intervals, $\times 200$. B Elytron, fragment from middle region showing two furrows, $\times 200$. C Mandibles, $\times 80$. D Antenna, terminal and subterminal joints, $\times 80$. E *Blatta orientalis* L. fragment showing reticulate ridging, $\times 200$.

tervals between the rows about 40 to 60 μ broad with a single median row of setal scars about 5 μ diameter and separated by about 20 to 40 μ : surface smooth and impunctate. (Fig. 5.B.)

Prothorax, head and other regions: coarsely punctate, punctures numerous, contiguous or separated by up to about 20 μ , very shallow, circular oval or irregular about 20 to 30 μ diameter, each enclosing an eccentric setal scar: intervals smooth and impunctate. (Fig. 5.C.)

Antennae: terminal joint rounded-conical about 160 by 100 μ and 50 μ diameter at the base, surface sparsely finely hairy, subterminal joint sometimes attached, obconical of similar dimensions. (Fig. 5.D.)

Mandibles: biting edge about 50μ long with two prominent closely set teeth. (Fig. 5.A.)

Hairs: setae, about 80 to 100μ long, slender and thin walled but otherwise similar to those of *S. paniceum*; clothing hairs lacking from the body. (Fig. 5.E.)

BLATTA ORIENTALIS L.

Elytra lacking. All sclerites reticulately ridged, the ridges enclosing more or less regular hexagonal areas about 10 to 15μ long, some having a minute pore in one angle; setal scars, scattered at intervals of about 70 to 100μ , each about 5 to 15μ diameter and sometimes surrounded by a circular unridged area.

Setae, about 80 to 100μ long, conical acuminate and thick-walled. Clothing hairs lacking. (Fig. 4.E.)

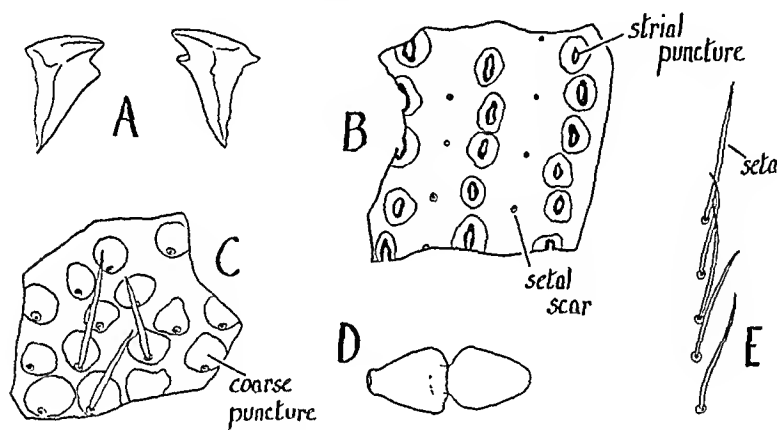


Fig. 5. *Lyctus brunneus* Steph.

- A. Biting edge of mandibles, $\times 80$. B. Elytron, fragment from middle region, $\times 200$. C. Prothorax, fragment showing coarse shallow punctures with enclosed setae or setal scars, $\times 200$. D. Antenna, terminal and subterminal joints, $\times 80$. E. Setae from elytron of whole insect, $\times 200$.

SUMMARY

1. A rapid and effective method of isolating insect fragments from powdered infested drugs is described.

2. The method depends on solution of the crude fibre of the drug in acetic anhydride containing 10 per cent. v/v of concentrated sulphuric acid.

3. The diagnostic microscopical characters of five of the more common beetle pests of drugs are described and illustrated.

In conclusion I wish to express my thanks to Mr. W. D. Hincks, M.P.S., F.R.E.S., of the Manchester Museum, for his assistance in confirming the identity of the species described.

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4. Hinton. *Bull. ent. Res.*, 1941, 31, 340.

DISCUSSION

THE CHAIRMAN (Dr. Norman Evers) said that many people who had to deal with foods had been disturbed by the stringent regulations introduced in the U.S.A. for the absence of insect fragments, rodents' hairs, and so on, and he was afraid that few drugs in this country would pass. Nevertheless, he felt that it might be necessary to have before long, on the basis of a method such as the author had described, some such requirements for drugs.

DR. T. E. WALLIS (London) said that many of these small beetles were quite difficult to distinguish when entire, and the difficulty was much greater in the powdered form, when examination of fine details of the form and attachment of the setæ and hairs and fine markings on the exoskeleton was required. Dr. Melville's work made it possible to distinguish, and frequently to name, the insects with which a powder was infested. He asked the author how far it was possible to see these insect particles in the crude fibre.

DR. D. C. GARRATT (Nottingham) said that the quality of infestation was as important as the quantity. The American regulations applied to foodstuffs and spices, and it could not be long before they were extended to drugs. Kent-Jones and his co-workers in this country, in their work on cereals, had dealt with rodent hairs, but could not give any authoritative help on insect fragments. Dr. Melville's paper would be of considerable value in that connection. He asked the author whether, in clearing for the detection of insect fragments, the rodent hairs would be lost; if so two separate methods of separation would be required.

MR. H. DEANE (Long Melford) said that, in his own experience, moths were more troublesome than beetles in stored drugs owing to their greater rapidity of reproduction. Moths were softer than beetles, and fewer fragments would be left with this treatment. Had Dr. Melville done any work on that subject?

DR. J. M. ROWSON (London) asked whether the possibility had been considered of extending the work from the qualitative to the quantitative field, also whether Dr. Melville considered that the B.P. requirement of complete absence of insect fragments, and also of animal matter and animal excreta, was too stringent.

MR. T. C. DENSTON (London) asked what drugs had proved to be unsatisfactory for examination by the flotation method. In view of the considerable use of one or other modification of this method in the U.S.A. he was a little surprised to find that it had been discarded.

MR. R. MAXWELL SAVAGE (London) drew attention to recent work on the separation of lice from animal skins.

MR. V. REED (London) asked whether there was any easy way of preventing the beetles from getting into the drugs.

DR. G. E. FOSTER (Dartford) asked which fragments could be considered most characteristic of infestation. How much of the beetle should be found in order to be sure that beetle infestation had actually occurred?

DR. COLIN MELVILLE, in reply, said that the detection of the particles in the crude fibre depended on the extent of the infestation and on the bulk of the crude fibre. In ginger he had been able to see the particles in the crude fibre, which was relatively small in amount, but with the more lignified roots or some of the herbs he had not been able to see the particles in the crude fibre, but had found them after extraction. Rodent hairs would be lost by his method of clearing, because they would be soluble in caustic soda, but it might be possible to apply the method directly to the drug without the preliminary clearing. He thought that then the rodent hairs would stand up to the acetolytic treatment, but a rather higher residue might be found, due to the non-cellulosic matter of the drug, which might not be dissolved under acetolysis. He thought, however, that it should be possible to find the rodent hairs. He hoped to undertake that problem when he had more time. He had not yet done any work on moths, other than looking at the larvæ. He imagined that the majority of moths would leave the drug after they had laid their eggs, so that to detect infestation one would have to look for the larvæ or larval skins which were cast when the larvæ changed to moths. He had found larval skins, but he had confined the paper to beetles, because he hoped to do further work on the larvæ of beetles and moths. He thought that a quantitative method could be worked out on the area of some segment such as the elytron. As most of these insects did not differ very greatly in weight, one could take the area of the elytron as being more or less a standard figure. He had always been doubtful about the B.P. requirements that drugs should be entirely free from insects because one could always find insects in powdered drugs. Hyoscyamus frequently contained insects which were not pests, but which were associated with the growing plant, and they would, of course, pass into the powder. Flotation methods were definitely unsatisfactory with herbs. At the interface of the two liquids one got a high percentage of the powdered herb, and it was very difficult to get complete separation. In any flotation method some insect fragments remained in the sediment of the drug and would not float to the surface at the interface. For the prevention of infestation, cleanliness in storage, the removal of all spillage and storage in suitable containers were important. On a small scale, to remove infestation the best treatment was with a mixture of carbon tetrachloride and ethylene dichloride, or carbon disulphide alone. About 1 fl. ounce of those liquids was sufficient to fumigate about 10 cu. ft. of storage space. It could be placed on a wad of cotton wool at the top of the container, and the vapour, being heavier than air, sank through the drugs and killed any pests which might be present. On a large scale, fumigation by hydrocyanic acid or methyl bromide or an ethylene oxide-carbon dioxide mixture was used. The most characteristic fragment for identification was the elytron. As to how much would be detectable, 10 g. of a sample of nutmeg yielded by the process one beetle leg which was almost intact.

INDIAN HENBANE

PART I

BY J. L. FORSDIKE AND B. JOHNSON

From The Analytical Laboratory, Boots Pure Drug Co., Ltd.

Received July 1, 1949

DURING the war, and in the post-war years, a considerable quantity of henbane has been imported into this country from India. Amongst this material was some which differed in appearance and especially in microscopical characters from typical *Hyoscyamus niger*. This suggested the possibility that this henbane was derived from some other species of hyoscyamus and it was thought worth while to carry out some investigation of hyoscyamus from India; with a view to ascertaining its botanical origin and characters. As a result, it has become apparent that there are several types of henbane growing in India and Pakistan in addition to *H. niger*. These other types have, in the past, commonly been referred to the species *H. reticulatus*, but they include plants whose habits are quite different from the typical form of this species and which, moreover, vary amongst themselves. It would appear, therefore, that there are a number of species of hyoscyamus growing wild in India, which might be collected as henbane. The question of the true botanical identity of these various species is still under consideration and it is hoped that more definite information will be available later.

The present paper is devoted to a description of one such species of hyoscyamus which grows wild in the North-West Frontier Province. This plant differs in habit from *H. niger* chiefly in the shape of its leaves, which are broadly ovate and much less indented (Fig. 1 and 2, A). It is hoped, in subsequent communications, to describe some further species of hyoscyamus which grow wild in India.

MATERIAL

The following description is based on six dried specimens, numbered H1 to H6, collected in Pakistan, in the area of the North-West Frontier Province, by Professor Qazilbash, of Islamia College, Peshawar, and on plants (H7) grown under glass at Lenton Experimental Station, Nottingham, from seeds sent by Professor Qazilbash.

For comparison, seven specimens of *Hyoscyamus niger* were used. These were:—

- N1. Collected at Chelsea Physic Garden, 1937.
- N2. Cultivated at Long Melford, 1948.
- N3. Cultivated at Hitchin, 1947.
- N4. Grown at Lenton, 1948.
- N5. Cultivated in the North-West Frontier Province, 1948.
- N6. From the Department of Botany, Government of Nepal, Katmandu, 1947.
- N7. Cultivated in India, supplied by the Divisional Forest Officer, 1947.

MICROSCOPICAL CHARACTERS

The plant is an erect, branching annual, 1 to 2 feet high. When dry, it has an odour resembling that of *Hyoscyamus niger* and a slightly bitter taste.

Stem. Cylindrical and smooth in the fresh state, but when dry flattened, longitudinally striated and usually showing one or more longitudinal furrows; light green, 2 to 5 mm. in diameter, slightly swollen at the nodes and clothed with white viscid hairs, up to 7 mm. long; leaf scars alternate. The transversely cut surface shows the remains of the pith with a central hollow, this is surrounded by a whitish ring of radiate xylem and a narrow, green bark.

Leaves. Ovate to oblong, 3 to 10 cm. long and 1.5 to 5 cm. broad, the lower two or three with petioles about a quarter the length of the leaf, the higher ones sessile, the uppermost clasping the stem; outline varying from entire to coarsely toothed, there being from 1 to 5 teeth on each side of the leaf, usually more on one side than on the other; apex acute, base acute in the lower leaves, obtuse to cordate in the upper; venation pinnate, with a broad midrib and 5 or 6 secondary veins on each side, which make wide angles with the midrib and terminate in the teeth where these are present or, if teeth are lacking, anastomose near the margin (Fig. 2, A).

The surface is covered with long white trichomes and has a viscid feel, especially when fresh. The dried leaves are thin and brittle, usually crumpled and broken; they are yellowish to greyish green in colour, the uppermost sometimes with a purple tinge.

Flowers. Typically solanaceous, the lower solitary in the axils of the leaves, the upper in a one-sided spike rolled back before flowering; about 20 mm. long with a pedicel 2 to 3 mm. long. Calyx urceolate, 10 to 15 mm. long and 4 to 8 mm. wide, having 5 lobes, each with an apical spine and 10 main veins; green, very hairy and persistent. Corolla funnel-shaped, 15 to 20 mm. long and 15 to 20 mm. in diameter at the mouth, the 5 lobes rounded and slightly unequal, very thin, pale buff with very prominent purple veins. Androecium of 5 free stamens, 2 long, about 12 mm. and 3 short, 8 to 10 mm., attached to the base of the corolla. Filaments very hairy in the lower half. Anthers 2 mm. long, black and dehiscing longitudinally. Ovary superior, and bilocular, consisting of 2 united carpels, smooth, cream-coloured, globular, about 2 mm. in diameter; it contains numerous ovules, showing axile placentation. Style filiform; stigma bilobed. Floral formula $K(5).C(5).A5.G(2)$.

Fruit. An ovoid-oblong pyxis, 12 to 15 mm. long, green, surrounded by the calyx (Fig. 2, C), which becomes larger in size, thicker and more coriaceous in texture after fertilisation, with 5 broad, shortly pointed lobes protruding above the fruit, which contains numerous seeds.

Seeds. Reniform and flattened, 1 to 1.5 mm. in diameter and 0.5 mm. thick; immature seeds green, mature seeds light brown. Testa finely reticulate, having 12 to 16 reticulations across the flat surface of the seed; the walls of the reticulations corrugated and the surfaces of the

INDIAN HENBANE

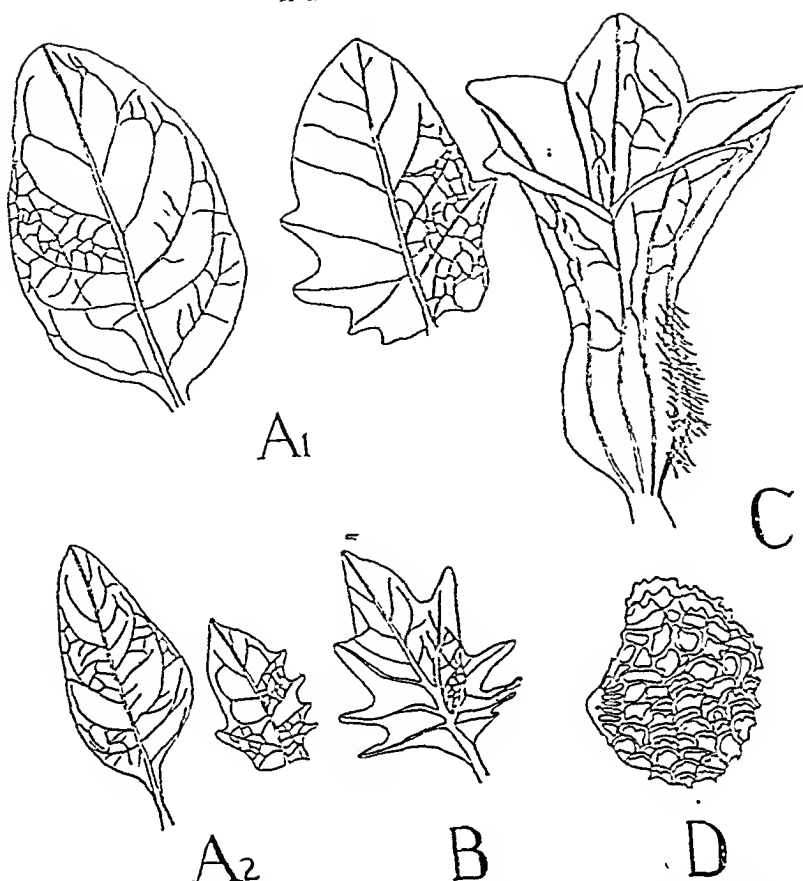


FIG. 2. A.1. Whole leaves, upper surface, natural size. A.2. Whole leaves, lower surface, natural size. B. *Hyoscyamus niger* leaf, natural size. C. Fruit $\times 4$. D. Seed $\times 20$.

depressions rugose (Fig. 2, D). The seed has a single scar of attachment to the placenta on one side. The weight of 100 seeds is 0.06 to 0.07 grammes.

MICROSCOPICAL CHARACTERS

STEM. Epidermis. Longitudinal rows of tetra- to hexagonal, tabular cells, 75 to 400 μ long, 70 to 155 μ wide and 25 to 55 μ high. Outer walls 5 to 10 μ thick, stratified and showing faint cuticular striations, parallel to the long axis of the stem; inner walls also thickened; anticlinal walls straight, thin, with occasional simple pits. Stomata rare, similar to those of the leaf, but lying at the same level as the epidermal cells. Trichomes similar to those of the leaf. Crystals absent.

Cortex. Consist of one outermost layer of ordinary parenchyma cells, containing chloroplasts; about 5 layers of collenchyma, averaging 45 μ radially, 60 μ tangentially, and 200 to 500 μ longitudinally, occasional cells very elongated, resembling fibres; about 4 layers of colourless parenchyma, showing intercellular spaces.

The endodermis consist of 2 layers of cylindrical cells with the axis directed longitudinally, containing starch grains 1 to 5 microns in diameter, mostly simple but occasionally 2 to 4 compound. All the cells of the cortex have cellulose walls.

Pericycle. Consists of 1 or 2 layers of common parenchyma with, on the outside of the primary phloem, small groups of fibres, measuring 10 to 20 μ radially, 25 to 50 μ tangentially, and up to 5000 μ longitudinally, with only slightly lignified walls. Fibres are lacking in the young stem.

Phloem. Primary phloem endarch, in bundles made up of groups of sieve tubes and companion cells, embedded in phloem parenchyma. The secondary phloem forms a continuous ring. Sieve tubes 12 to 16 μ in diameter, transverse walls oblique, sieve plates and callus evident. Companion cells 3 to 4 μ in diameter. All cells with cellulose walls.

Cambium. Consists of 1 or 2 layers of very thin-walled cells, tetragonal in transverse section. Average dimensions, radial 4 μ , tangential 12 μ , longitudinal 60 μ .

Xylem. Primary xylem endarch, in groups opposite the primary phloem; the secondary xylem forming a continuous ring. Consists of annular, spiral, reticulate and pitted vessels, 15 to 75 μ in diameter; wood parenchyma cells, usually 5-sided with pitted walls, averaging 15 μ radially and 20 μ tangentially and wood fibres, with oblique slit-shaped pits, about 20 μ in diameter. Medullary rays, uni- to triseriate, consisting of tangentially elongated pitted parenchyma cells. All elements of the xylem have lignified walls; intercellular spaces absent.

Pith. Consists of large, thin-walled parenchyma cells with cellulose walls and showing numerous intercellular spaces, which usually appear triangular in transverse section. At the periphery, a ring of phloem bundles, similar in structure to the normal phloem and having small groups of fibres, resembling the pericyclic fibres, on the inner side.

LEAF. Upper epidermis. Epidermal cells of the interneural region 100 to 200 μ long by 50 to 100 μ wide by 20 to 40 μ high, with sinuate anticlinal walls and a thin cuticle. Epidermal cells of the main veins straight-walled and elongated in the direction of the vein, 150 to 400 μ long by 25 to 50 μ wide by 20 to 40 μ high, (Fig. 3, B, ep. 1) the cells bearing the hairs are higher and wider than the normal epidermal cells and have rounded edges. Stomata 35 to 65/sq. mm., averaging 40 by 22 μ , lying slightly above the level of the epidermal cells and surrounded by 3 to 5 subsidiary cells, one of which is markedly smaller than the others. Stomata absent over the midrib and the primary veins. Covering trichomes numerous, conical, uniseriate, of 2 to 4 cells, 75 to 290 μ long (Fig. 3, D, c). Glandular trichomes fairly numerous, especially on the veins, 450 to 750 μ long, with a uniseriate stalk of 2 to 6 cells, showing cuticular reticulations and a multicellular, glandular head (Fig. 3, D, gl). On the veins, in addition to the above types, are some very large trichomes, up to 7000 μ long and containing up to 12 cells; the basal cells being 500 to 1400 μ long by 40 to 160 μ wide; the apical cell sometimes forming a rudimentary gland (Fig. 3, E).

Lower epidermis. (Fig. 3, A and B, ep. 2). Very similar to the upper,

INDIAN HENBANE



FIG. 1. Entire plant, grown at Nottingham. One-third natural size.

but the stomata are more numerous, 40 to 112/sq. mm. and slightly larger, 45 by 30μ . The trichomes are also more numerous.

Mesophyll. On the upper side of the leaf, one row of palisade cells, containing chloroplasts; cells averaging 85μ high by 20μ wide (Fig. 3, B, p); chloroplasts 2 to 4μ in diameter. There are large intercellular spaces, especially under the stomata.

The spongy mesophyll consists of 3 or 4 layers of more or less stellate cells, 24 to 30μ in diameter, with large intercellular spaces (Fig. 3, B, sp). Idioblasts in the upper layer contain calcium oxalate crystals of three kinds:—

(a) Tetragonal prisms (Fig. 3, F, pr.).

(b) Cluster crystals. These are commonly composed of a small number of prisms. A type consisting of single large prism with a number of smaller ones attached is of frequent occurrence (Fig. 3, F, cl.).

(c) Microsphenoidal crystals.

The prisms and clusters are 4 to 48μ long by 3 to 26μ wide.

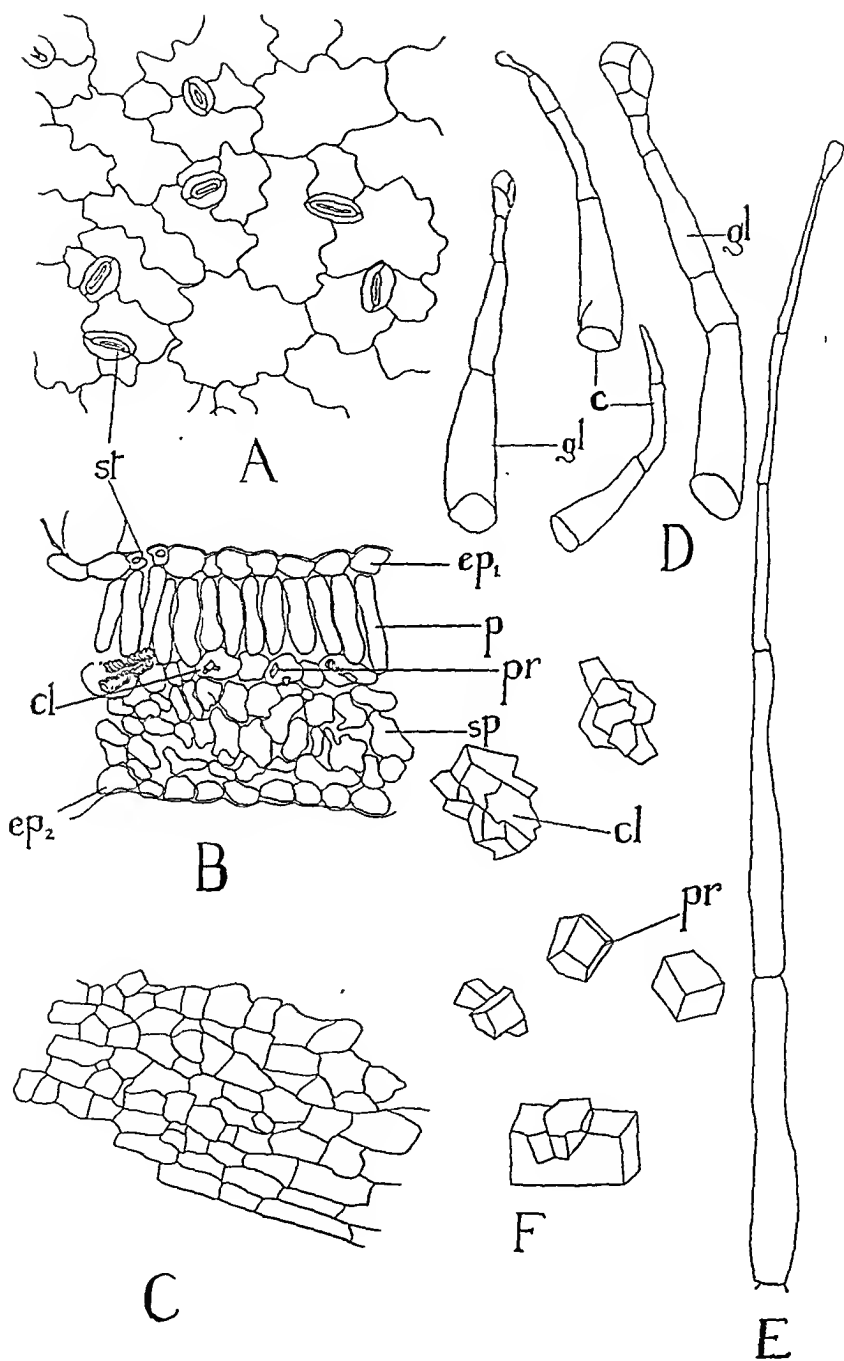
Midrib. There is 1 layer of collenchyma on the upper surface and 1 to 3 layers on the lower; cells about 30μ in diameter and up to 500μ long. Within the collenchyma is colourless parenchyma, about 10 layers on the upper and 4 on the under side, with large intercellular spaces; the cells sometimes containing crystals of calcium oxalate. Cells average 60μ in diameter by 200 to 400μ long. Endodermis of 1 or 2 layers of cells with slightly thickened cellulose walls, containing starch grains and chloroplasts.

Meristele. In the form of an arc, made up of 8 to 10 simple, collateral vascular bundles separated by uni- or biseriate medullary rays. Xylem of 7 to 8 layers consisting of annular, spiral and reticulate vessels, 15 to 24μ in diameter, embedded in xylem parenchyma; all elements lignified. Phloem consisting of sieve tubes and companion cells, embedded in phloem parenchyma. Perimedullary phloem, in groups corresponding roughly in number and position with the vascular bundles, and in form similar to the bundle phloem, is also present. The pericycle consists of 3 or 4 rows of somewhat thickened, fibre-like cells, 10 to 25μ in diameter and 200 to 300μ long, with cellulose walls.

FLOWER. *Calyx*. *Upper epidermis*. The basal cells are rectangular, with straight anticlinal walls, 15 to 25μ long, 15 to 25μ wide and 15 to 25μ high. Upwards the cells become larger, 30 to 65μ long, 15 to 30μ wide and 15 to 25μ high, the walls become more sinuate and the shape irregular, except over the veins, where the rectangular shape is preserved. Towards the top the cells are again smaller. The epidermis has a cuticle about 4μ thick which, over the main veins, sometimes shows cuticular striations, running parallel with the direction of the vein. The stomata increase in number towards the top of the calyx, where the number per sq. mm. is 80. Each stoma is surrounded by 3 subsidiary cells. The trichomes are very numerous and similar to those of the leaf.

Lower epidermis. Similar to the upper (Fig. 3, C).

Mesophyll. Consists of 4 to 6 layers of thin-walled parenchyma cells,



containing chloroplasts ; cells more or less stellate in form, averaging 25μ in diameter, with very large intercellular spaces. Idioblasts filled with microsphenoidal crystals of calcium oxalate fairly numerous towards the base of the calyx only ; prisms and cluster crystals absent.

Meristeles. These are similar to the meristeles of the leaf.

Corolla. Upper epidermis. At the base, the epidermal cells are elongated and rectangular in shape, 35 to 90μ long, by 15 to 30μ wide by about 20μ high ; the anticlinal walls are slightly wavy and the outer walls have a fairly thin, striated cuticle. Towards the apex, the cells become smaller and the anticlinal walls sinuate. Stomata very rare ; trichomes absent. *Lower epidermis.* Cells similar in shape to those of the upper epidermis, but slightly larger. Trichomes present, of two kinds :—(a) Short, conical, covering trichomes, 1 to 4 cells long. (b) Glandular trichomes, almost confined to the veins in the lower part of the corolla ; they have a 1- or 2-celled stalk and a well-developed multicellular glandular head.

Mesophyll. Consists of 4 or 5 layers of spongy parenchyma. Cells stellate, with large intercellular spaces.

Stamen. Filament. Epidermal cells elongated-rectangular with straight walls ; no stomata. Trichomes of 3 kinds are present :—(a) With a uniseriate stalk and a multicellular glandular head. (b) Covering trichomes, similar to those of the leaf. (c) Capitate trichomes, having a long apical cell. The mesophyll consists of about 5 layers of thin-walled parenchyma cells, with a central vascular strand containing lignified spiral vessels.

Anther. Epidermis of irregular cells with a few stomata. Mesophyll of ordinary parenchyma cells with fairly large intercellular spaces. The meristeles consist entirely of xylem, which contains lignified spiral vessels. There is a fibrous layer of 2 to 3 layers of cells, near the connective.

Pollen. The pollen grains have 3 pores and an irregularly pitted exine, diameter 45 to 55μ ; diameter of pores 10 to 15μ .

Pistil. Ovary. The epidermal cells of both surfaces are tetragonal, stomata rare. The mesophyll consists of about 10 layers of closely packed parenchyma cells, without intercellular spaces. The meristeles consist wholly of xylem, made up of spiral vessels, the walls of which are incompletely lignified.

Septum and Placentæ. Epidermis similar to that of the ovary wall ; mesophyll of spongy parenchyma with large intercellular spaces.

FRUIT. Pericarp. Outer epidermis. More or less tetragonal, tabular cells 55 to 95μ long, 15 to 30μ wide and 15 to 22μ high ; anticlinal walls thin and slightly wavy, outer walls with a very thin cuticle. Trichomes absent.

FIG. 3. A. Lower epidermis of leaf, surface view. B. Transverse section of interneural lamina of leaf. C. Lower epidermis of calyx, near base, surface view. D. Glandular and covering trichomes from the leaf. E. Very long trichome from midrib of leaf. F. Calcium oxalate crystals. c, covering trichomes ; cl, cluster crystals ; ep₁, upper epidermis ; ep₂, lower epidermis ; gl, glandular trichomes ; p, palisade layer ; pr, prismatic crystals ; sp, spongy mesophyll ; st, stomata. F $\times 300$, remainder $\times 150$.

Inner epidermis. Tabular cells, 75 to 130 μ long, 20 to 40 μ wide and 20 to 26 μ high, anticlinal walls thin and wavy.

Mesophyll. Thin-walled, stellate cells, 13 to 26 μ in diameter and 40 to 75 μ long, with large intercellular spaces. Contains numerous vascular strands, each consisting of 1 to 3 spiral vessels, 4 to 12 μ in diameter, with lignified walls. The parenchyma cells are filled with starch grains, polyhedral to subspherical in shape, 2 to 10 μ in diameter, mostly simple, but including occasional compound grains of 2 to 4 components.

Septum and Placentæ. Epidermis similar to the inner epidermis of the pericarp; mesophyll of thin-walled, stellate parenchyma, 30 to 60 μ in diameter and 75 to 200 μ long, containing starch similar to that in the pericarp.

QUANTITATIVE DATA

Palisade ratio. The palisade ratio of the material under examination is from 4.25 to 5.5. This range is exactly the same as that found for *H. niger*.

Stomatal index. The range of stomatal index of the upper surface was from 19.2 to 24.0 and of the lower surface from 18.7 to 26.0. These figures compare with values of 21.0 to 22.5 for the upper surface of *H. niger* and 22.1 to 24.8 for the lower surface given by Rowson.¹

Vein-islet number. The vein-islet numbers found for the species investigated ranged from 4.5 to 15.5; the range for *H. niger* was 6 to 16.

Calcium oxalate crystals. The prisms and cluster crystals in our unnamed species were, in general, larger than those of *H. niger* and this feature provides the best means of distinguishing the two species when broken or in the form of powder. In Table I are shown the ranges of sizes of the crystals in all the specimens examined and also the percentages of crystals in each sample which exceeded 25 μ in length.

TABLE I
DIMENSIONS OF CALCIUM OXALATE CRYSTALS

DIMENSIONS OF CALCIUM OXALATE CRYSTALS

Specimen	Size		Number less than 25 μ long	Number greater than 25 μ long	Percentage greater than 25 μ long
	Minimum μ	Maximum μ			
<i>Unnamed Species</i> :—					
H.1... ..	6	42	390	120	23.6
H.2... ..	6	40	308	104	25.4
H.3... ..	4	40	352	146	24.4
H.4... ..	5	40	498	196	28.3
H.5... ..	6	45	317	142	31.0
H.6... ..	4	45	384	138	26.0
H.7... ..	4	48	360	220	38.0
All specimens ...	4	48	2609	1066	29.0
<i>H. niger</i> :—					
N.1... ..	5	35	364	37	9.2
N.2... ..	4	32	591	49	7.5
N.3... ..	4	28	549	37	6.3
N.4... ..	4	26	460	5	1.1
N.5... ..	6	32	480	20	4.0
N.6... ..	5	26	410	18	4.2
N.7... ..	5	30	374	15	3.8
All specimens ...	4	35	3228	181	5.3

INDIAN HENBANE

These figures were obtained by the examination of powders, prepared from representative samples of the specimens. It was observed that, in both species, the size of the crystals varied markedly from leaf to leaf, one leaf containing large crystals and another smaller ones while, from some leaves, crystals were entirely absent. It is thus clear that no reliance, from this point of view, can be placed on the examination of single leaves, but, by examining representative samples of powders it was found that, while the crystals of *H. niger* never exceeded 35μ in length, and except in one specimen, did not exceed 32μ , all specimens of the Indian material contained crystals up to 40μ long and some up to 48μ . Moreover, the percentage of crystals exceeding 25μ in *H. niger* was never greater than 10 and averaged only about 5, while in the Indian material it ranged from 23.6 to 38.0 per cent.

Alkaloid contents. The content of total alkaloids, calculated as hyoscyamine, of the 6 samples, H1 to H6, grown in India, are shown in Table II. These were determined by the method of the British Pharmacopœia for *Hyoscyamus*.

TABLE II
ALKALOID CONTENTS

Specimen				Total alkaloids, as Hyoscyamine	Specimen				Total alkaloids, as Hyoscyamine
				per cent.					per cent.
H.1	0.038	H.4	0.034
H.2	0.038	H.5	0.046
H.3	0.031	H.6	0.055

Ash values. In Table III are shown the results of determinations of total and acid-insoluble ash, by the methods of the B.P., on the material under investigation and the amounts of ash soluble in dilute hydrochloric acid, obtained by difference.

The new material thus has a soluble ash ranging from 15.7 to 18.3 per cent. This quantity is distinctly higher than is normally found in

TABLE III
ASH VALUES

Specimen				Total Ash	Acid-insoluble Ash				Acid-soluble Ash
				per cent.					per cent.
<i>Unnamed Species :—</i>					<i>per cent.</i>				
H.1	21.5	4.9				16.6
H.2	21.5	4.8				16.7
H.3	22.3	4.6				17.7
H.4	22.7	4.4				18.3
H.5	23.5	6.1				17.4
H.6	21.7	6.0				15.7
H.7	20.2	3.2				17.0
<i>H. niger :—</i>									
N.1	10.0	3.0				7.0
N.2	13.7	1.7				12.0
N.3	15.3	1.3				14.0
N.4	16.0	4.6				11.4
N.5	19.8	3.6				16.2
N.6	21.7	3.3				18.4
N.7	25.1	6.0				19.1

H. niger grown in England. The 3 samples of *H. niger* obtained from India, however, had soluble ash up to or above this range.

GERMINATION

Hyoscyamus seed is known to be difficult and erratic in germination. For this reason a germination test was carried out on the Indian seed. Three methods of preliminary treatment were tried:—(A) The seed was placed in the refrigerator, at approximately 5°C., overnight; (B) The seed was soaked in a solution of hydrogen peroxide, approximately 2 per cent. w/v, for 18 to 24 hours. This method of treatment was suggested for belladonna seed by Sievers²; (C) The seed was soaked in concentrated sulphuric acid for 2½ minutes and then washed with water. This method is recommended by Newcomb and Haynes³.

The seeds so treated, together with untreated seed (D) were sown in pans on 30th July, 1948. Fifty seeds were put in each pan and 2 pans were sown with each type of seed. John Innes Seed Compost was used and the pans placed in the greenhouse. Counts were made after 10 and 17 days.

The results are recorded in Table IV.

TABLE IV
GERMINATION OF INDIAN HENBANE SEED

Pan						1st Count	2nd Count	Average of Two Pans 2nd Count	Germination
									per cent.
A. 1	2	2	2.5	5
A. 2	1	3		
B. 1	35	40	26.0	52
B. 2	9	12		
C. 1	1	2	1.5	3
C. 2	1	1		
D. 1	3	5	4.0	8
D. 2	3	3		

The results show that the treatment with hydrogen peroxide had a marked effect in stimulating germination. The other two treatments, that with sulphuric acid and refrigeration actually produced inferior germination to that obtained with the untreated seed. As regards the sulphuric acid treatment, this confirms the experience of Sievers² with belladonna seed. He states that treatment with sulphuric acid was of little use. It is probable that the period of refrigeration used in this experiment was too short. Melville and Metcalfe⁴ found refrigeration successful in stimulating the germination of belladonna seed, but they used periods of 7 or 14 days. It is hoped to try their method with hyoscyamus seed this year.

The plants reached the flowering stage in 8 weeks after sowing and were then gathered and either dried or preserved in alcohol for examination.

SUMMARY

1. A type of *hyoscyamus* growing wild in India is described. The species to which this plant should be referred has not yet been determined.

2. This plant may be distinguished from *H. niger*, when unground, by the shape of the leaves, which are ovate to oblong and much less indented. The powder is distinguishable because the calcium oxalate crystals present are larger than those in *H. niger*; the presence of any calcium oxalate crystals exceeding 35μ long, or of more than 10 per cent. exceeding 25μ may be taken as indicating something other than true *Hyoscyamus niger*.

3. The total alkaloid contents of the specimens examined were, except in one case, less than the minimum required by the British Pharmacopœia for *Hyoscyamus*.

4. The acid-soluble ash was greater than that usually found in *H. niger* grown in England, but about equal to that found in *H. niger* from India.

5. A report of a germination test on the seeds is given. It was found that soaking the seeds for 18 to 24 hours in 2 per cent. w/v solution of hydrogen peroxide before sowing gave the best results.

Our thanks are due to Professor Qazilbash, of Islamia College, Peshawar and to Mr. K. C. Chatterjee, of Boots Pure Drug Company (India), Ltd., for the provision of material; to Mr. A. W. Billitt, of Lenton Experimental Station, Nottingham, for carrying out the germination test and for growing the plants; to Dr. R. Melville, of the Royal Botanic Gardens, Kew, for information; and to Mr. H. O. Meek, for helpful criticism. We are indebted to the Directors of Boots Pure Drug Company, for permission to publish this paper.

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1. Rowson, *Quart. J. Pharm. Pharmacol.*, 1946, 19, 140.
2. Sievers, *Amer. J. Pharm.*, 1914, 86, 483.
3. Newcomb and Haynes, *Amer. J. Pharm.*, 1916, 88, 1.
4. Melville and Metcalfe, *Pharm. J.*, 1941, 146, 116.

DISCUSSION

MISS B. JOHNSON read a summary of the paper.

DR. T. E. WALLIS (London) said that the authors seemed to indicate that this plant was different from *Hyoscyamus niger* or the other known species of *Hyoscyamus*, but he hoped they would be very careful in future work on the same material in deciding that it really was something different. Experience with plants in general showed to what an enormous extent details of structure varied in the same plant. The most extraordinary differences in the leaves of plants known to be of the same species might be found. He thought the shape of the calcium oxalate crystals was more important than their size. It was known that the size of crystals in henbane and similar plants varied enormously. Unless a careful review was made of their size, one might arrive at an inaccurate result. This plant closely resembled annual henbane, and it might better be compared with henbane generally than with *Hyoscyamus niger*.

DR. J. M. ROWSON (London) said that he had been interested in the

remarks on the action of hydrogen peroxide on the germination of seeds. He had himself had some experience of the erratic nature of the germination of *Hyoscyamus* seeds. He also was sceptical as to whether this was a different species of *Hyoscyamus*. He had grown annual henbane for the last ten years, with the same strain of seed and it was amazing to note the variation which occurred. He could produce leaves identical with those figured in the paper, broadly ovate, or with just one marginal tooth. He did not think there was any difference in that respect, nor was there much difference in the crystal sizes and total ash. The low alkaloidal content was not of great significance. The alkaloidal content of *Hyoscyamus* varied greatly with the environment in which it was cultivated, and it was possible that here they had poor cultivation. If the authors had details of the alkaloidal content of the Nottingham-grown material, it would be interesting to see how that compared with the Indian samples.

DR. W. MITCHELL (London) asked if there had been any attempt to characterise the alkaloids. It would be interesting to know whether they differed from those of normal *Hyoscyamus*.

MR. A. R. G. CHAMINGS (Horsham) said that, in the cultivation of belladonna in Leicestershire, he had found that successive refrigeration of the seed was much better than a single refrigeration.

DR. C. MELVILLE (Manchester) said that the use of calcium oxalate crystal determinations for identification purposes was interesting, and this could be extended. The illustration of the trichomes from the leaf gave the impression that the head of the gland was relatively simple, composed of not more than eight or ten cells. In *Hyoscyamus niger* the gland often contained upwards of twenty cells, with frequently two cells at the apex, one of which extended or projected beyond its companion; so that if the glands on this *Hyoscyamus* were relatively simple, it might prove a characteristic for differentiating it from *Hyoscyamus niger*.

DR. G. E. FOSTER (Dartford) asked whether the authors had investigated the volatile alkaloidal content of the drug.

MISS JOHNSON said that they would give further consideration to the suggestion that the plant was possibly a variation of annual henbane. The amount of material had been so small that it had been impossible to do anything more than the ordinary alkaloidal determination and the volatile alkaloids had not been determined. Regarding germination, they had stored their seeds in the refrigerator overnight at 5°C. and as stated, that had given very poor results. Since the paper had been written, they had tried longer periods of refrigeration. They would now try successive periods. Unfortunately, they had not yet had any reports on the effect of longer refrigeration.

MR. FORSDIKE said they had consulted authorities in this country and in India, and were satisfied that their material represented a species different from *Hyoscyamus niger*; it was hoped before next year to give it a definite botanical name. They had found in commercial material, during the war in particular, a henbane which differed in a number of ways from the typical *Hyoscyamus niger*, and they were sure that more than one species of *Hyoscyamus* was represented.

VEGETABLE PURGATIVES—PART I

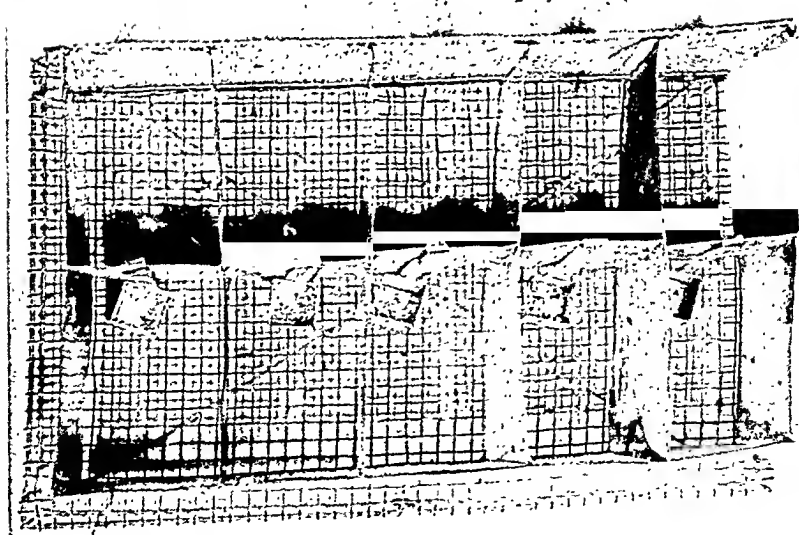
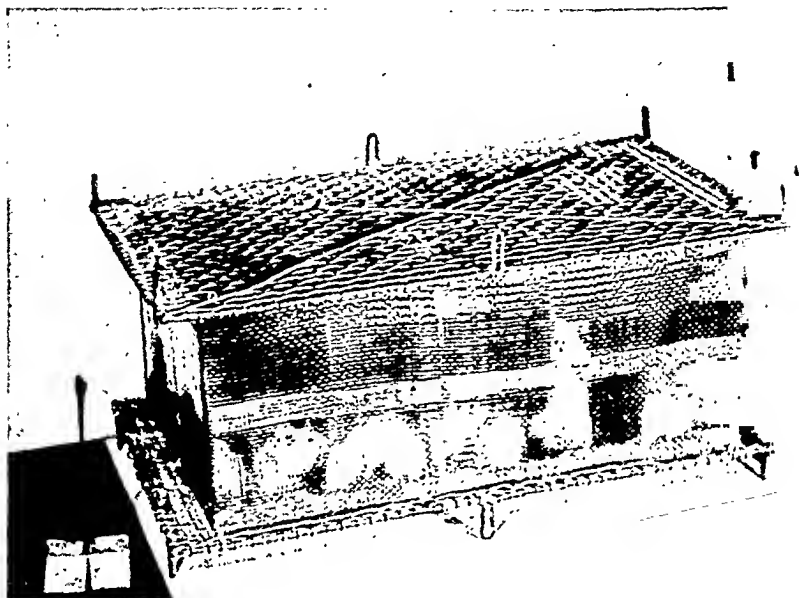


FIG. 1. Photographs of the cage. Upper: Side-view of the cage with a pair of food containers on the left. Lower: Top-view of the cage with the top grid removed.

THE BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES

PART I.—SENNA LEAF AND FRUIT AND THEIR PREPARATIONS

By T. C. LOU

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Received July 4, 1949

INTRODUCTION

As part of a general investigation on the vegetable purgatives containing anthracene derivatives, I was asked in 1947 to carry out biological assays of several of these drugs and their preparations. Several biological methods of assay have been described and reviewed by Munch¹ and Viehoveer². More recently, Loewe³ used rhesus monkeys and claimed that his method is applicable to many types of laxatives. Straub and Gebhardt⁴ used white mice and determined the minimum effective dose (ME) of senna infusions. The potency was expressed as the number of such doses per ml. of the infusion. Geiger⁵ adopted a similar method but instead of using animal units the use of a standard (a 5 per cent. infusion of senna leaf) was introduced. The potency of a test preparation was compared with the standard by comparing the percentage of mice which produce positive response. Later, Hazleton *et al.*^{6,7,8} introduced the term T.C.D. (Threshold Cathartic Dose—i.e. the dose which produces catharsis in approximately 50 per cent. of the mice), and Grote and Woods⁹ introduced the use of powdered senna leaf as their standard of reference. Collier *et al.*¹⁰ further modified the method by using the ratio of the number of unformed faeces (UFF) to the number of total faeces (TF) as the criterion of purgative activity.

The most promising method seemed to be that of Geiger as modified by others, since mice are convenient to handle and require only small amount of test material; this last point is particularly important when only small quantities of pure compounds are available for assay. Accordingly, work was commenced in 1947 on the basis of their methods; but experience soon showed that improvements were necessary for the following reasons: (a) the handling of large number of frog-jars or beakers used as mouse containers during test is inconvenient; (b) droplets of water occasionally appeared on the inside of the jars, indicating a high humidity due to bad ventilation; (c) it is impossible to use water-bulbs with the jars and previous workers have withheld drinking water during test, but this seems undesirable, since water plays a large part in purgation; (d) my experience showed that increase in dose of purgative produced a corresponding increase in the number of wet (or unformed) faeces (i.e. those which differ from the normal dry ones in being round and pasty and leaving a brown stain when placed on blotting paper). It appears therefore that a method based on a quantitative response, rather than the qualitative ("all or none") type used by Geiger and others, would give more accurate results. Collier¹⁰ apparently had a similar idea as he determined the ratio of UFF/TF and used this value as a criterion

of purgative activity. This method, however, is tedious as it involves counting a very large number of faeces in each test.

To overcome these defects, (a) special cages were devised to avoid the use of large number of frog-jars or beakers, (b) a definite proportion of water was added to the feeds during test, and (c) counts of *wet* faeces only were used as criteria of activity. The resulting method described in this paper is not only free from the defects already mentioned, but is more convenient in use and gives results of a comparatively high degree of accuracy. Furthermore, this method has been found applicable to other purgatives apart from senna; Hazleton *et al.*⁷ report that their method is unsuitable for cascara and aloes.

GENERAL EXPERIMENTAL DETAILS

1. *The Standard.* It was decided to use the powdered crude drug as standard, wherever possible, rather than infusions, because infusions may not contain the entire activity. Moreover, owing to the unavoidable variation in preparation, successive infusions may vary in potency. According to Collier¹⁰, an infusion made from 5 to 6 mg. of senna fruit (0.24 g./kg. body-weight) produced no response; I have found that the same dose of senna fruit given directly always produce marked response. While this may be due to my sample of senna fruit being highly active, it may also be due to the fact that the infusion (as made by Collier) does not contain all the activity. The standard, in powdered form, is kept in evacuated glass bottles in a refrigerator. To prepare a standard suspension for administration, a weighed amount of the powder is triturated in a mortar with a small quantity of boiling distilled water and made up to volume in a measuring cylinder with cold distilled water. For a mouse of 20 g. body-weight, a dose of 0.5 ml. is suitable.

2. *Design of the Cages.* The cage as shown in Figure 1 is 15 inches long, 9 inches wide, and 6 inches high. It is divided into 10 compartments with tinned plates. The outer walls were made of "window substitute" (wire gauze impregnated with transparent plastic). Each compartment has a food container made of tinned plate; these containers were connected in pairs by a \cap -shaped handle which hangs over the wall dividing two compartments. The floor and ceiling consist of loose (detachable) grids made of galvanised wire with a mesh of 1 cm. by 1 cm. Six feet are provided at the edges of each of these grids so that the bottom grid is raised about 1 inch from the table on which a sheet of white blotting paper is provided to receive the faeces. The advantages of this cage are (a) good ventilation is obtained, (b) the inconvenience of using a large number of jars is avoided and (c) the bottom grid can be easily removed (either for inspection of the adhering faeces or for washing) by inverting the cage; the top grid will now serve as a floor.

3. *The Test Animal and Diet.* White albino mice weighing not less than 18 g. are used in all tests. Owing to the variation in response of the sexes mentioned by Hazleton *et al.*⁸, male mice only are used. They are housed in metal cages and fed with rat-cubes (Diet 41 supplied by the Associated London Flour Millers Ltd.). In addition, each mouse receives an unrestricted supply of fresh tap-water; green vegetables are given over

BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES. PART I

the week-ends only, thus avoiding the possible interference with the test during the week. The animals, if in good condition, can be used repeatedly after a resting period of not less than 1 week.

4. *The Test.* All food and water are withdrawn from the mice early in the morning and the animals are put singly or in pairs into each compartment of the cage. After 2 or 3 hours, the faeces are examined and any mouse having soft or wet faeces is discarded. The mice are then weighed to the nearest gramme and the weights recorded. They are evenly divided into 4 groups each of 10 mice. As will be shown, later, it is necessary to give two dose-levels each of the standard preparation and the test preparation for every assay. The dose is given into the oesophagus of the animal by means of a blunted needle attached to a 1 ml.-syringe. After dosing, the animals are kept under observation for at least 12 hours.

During this testing period, a special food made by mixing 10 parts of powdered rat-cubes and 7 parts of fresh tap-water is supplied in the food containers. This moist food has several advantages: it allows the animals a uniform intake of water in proportion to the diet; it ensures the normal working of the alimentary canal during the 12 hours of test; it, unlike the dry rat-cubes, when scattered during feeding, does not absorb water from the wet faeces. Moreover, the inconvenience of using a large number of water-bulbs is avoided.

Purgation is indicated by the excretion of wet faeces which are recognised by their somewhat rounded shape and the presence of a brown stain surrounding each on the blotting paper. They can be easily distinguished from the normal dry faeces which are elongated in shape and do not stain the paper. Counting of the wet faeces is usually started from the second hour after dosing and repeated every 1½ hours until the fifth or sixth hour. The final counting is done early in the following morning.

RELATION BETWEEN DOSE AND RESPONSE

One would expect that an increase in the dose of purgative would result in an increase in the number of wet faeces produced; in other words, the response evoked by purgatives is "quantitative" rather than the "all or none" type such as that evoked by digitalis where the animal

TABLE I
NUMBERS OF WET FAECES PRODUCED BY GROUPS EACH OF 10 MICE

Experiment No	Body-weight of 10 Mice	Dose mg/kg	Number of WF per Group	Number of WF per kg. of Mouse
30	8			
	210	280	8	38.1
	245	350	15	61.2
	198	840	25	126.3
	233	1050	34	146.0
33	328	280	19	57.9
	305	350	23	75.4
	329	840	46	139.8
	320	1050	49	153.2

either lives or dies. The following experiments were designed to prove this assumption and to investigate the relation, if any, between dose and response.

4 graded doses of powdered senna leaf suspended in distilled water were given to 4 groups each of 10 mice and the number of wet faeces (WF) produced by each group was recorded as shown in Table I.

Table I clearly shows that an increase in dose produced an increase in the number of wet faeces. To determine the relation between the dose and response, graphs were constructed to illustrate the relation of response to (a) dose (Fig. 2) and (b) logarithm of the dose (Fig. 3). To eliminate

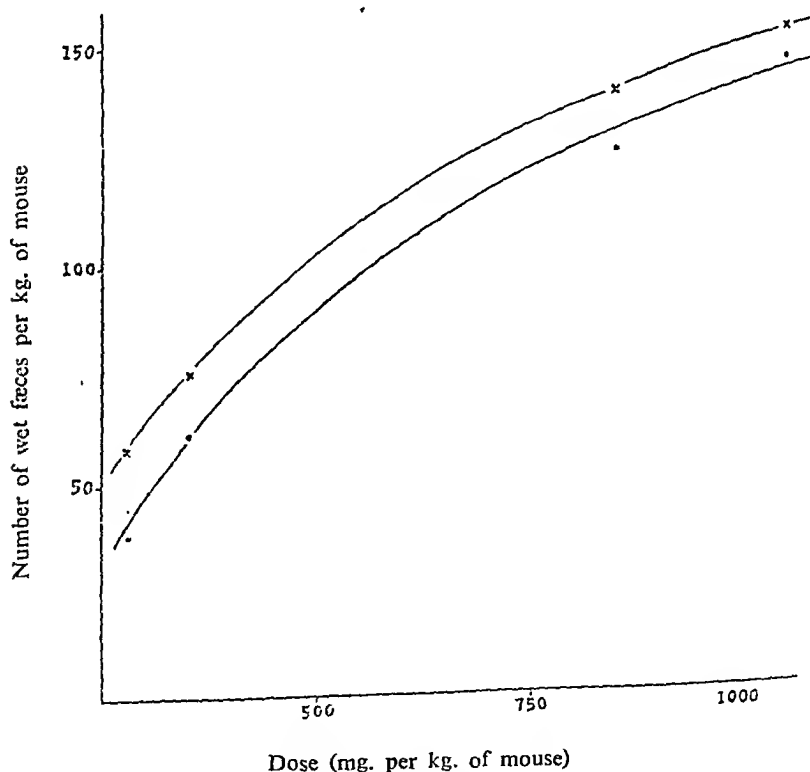


FIG. 2.—Relation of response to dose.

the variation in body-weight of different groups of mice, the response was expressed as number of wet faeces per kg. of mouse. These graphs show that the relation response/log. dose is linear, whereas response/dose is not so. This conclusion was confirmed by later experiments (using the same sample of senna leaf as above) involving 92×10 mice; the responses of groups of mice were averaged for each dose-level and the results are shown graphically in Fig. 4. It can be clearly seen that the response is proportional to the log. dose.

In Fig. 3, it will be noted that the log. dose/response lines representing

the same dose-levels given on different days are almost parallel. Though most subsequent experiments resulted in lines of similar slope this was not invariably true. Hence, it is necessary always to give two dose-levels each of the test and of the standard for every assay, so that the slope of the log. dose/response line for that particular day can be determined. Furthermore, if the distance between the lines of response is unusually great or the slopes of the log dose/response lines of the standard and the test given on the same day differ to a great extent, one may suspect that either the choice of the dose-levels is unsuitable or the nature of the response is different due to different types of active constituents present in the standard and the test, or that the distribution of mice is uneven. It may be necessary, therefore, to repeat the test after due consideration.

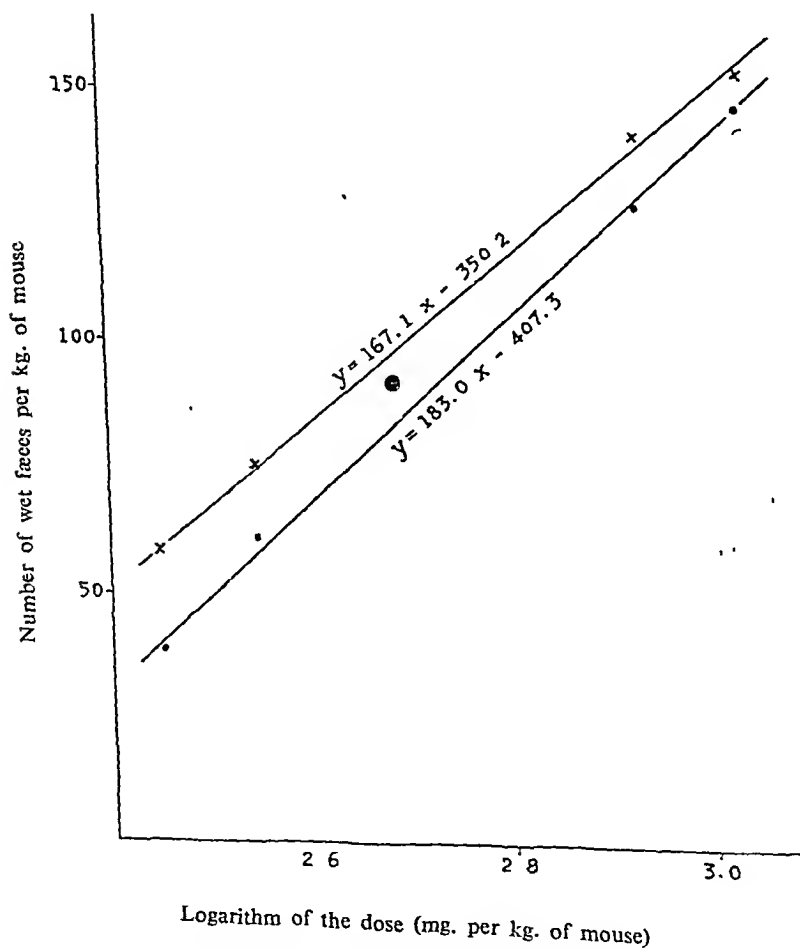


FIG. 3.—Relation of response to the logarithm of the dose.

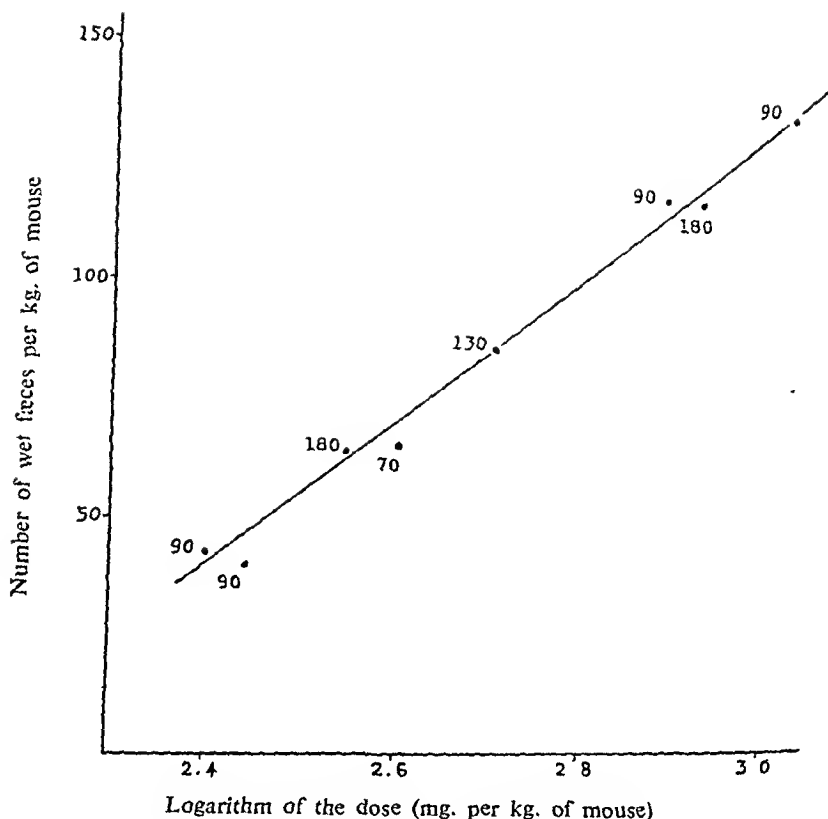


FIG. 4.—The average response/log. dose line. The figures indicate the number of mice used.

EXAMPLE OF THE METHOD: ASSAY OF DRY EXTRACT OF SENNA

A sample of dry extract of senna (E2) made in this laboratory was compared with the laboratory standard sample of senna fruit (Ps) from which the extract was made, by testing them on 4 groups each of 10 mice. In Table II are given the details of the test.

TABLE II
BIOLOGICAL ASSAY OF E2 (EXPERIMENT NO. 68)

	Body-weight of 10 Mice	Dose mg./kg.	Number of WF per Group of Mice	Number of WF per kg. of Mouse
Ps	197	350	21	106.7
Ps	186	1050	47	252.7
E2	190	150	28	147.4
E2	185	450	51	275.7

Calculation: (At length to demonstrate the principle involved).

Tripling the dose of Ps caused an increase of 146.0 WF/kg.

Tripling the dose of E2 caused an increase of 128.3 ..

BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES. PART 1

Mean effect of tripling the dose	=137.15	..
Mean effect of the two doses of Ps	=179.7	..
Mean effect of the two doses of E2	=211.55	..
Difference between the mean effects of Ps and E2			=31.85	..

Since the number of wet faeces per kg. of mouse is proportional to the logarithm of the dose

$$\frac{137.15}{\log. 3} = \frac{31.85}{\log. r} \quad \text{or} \quad \frac{137.15}{31.85} = \frac{\log. 3}{\log. r}$$

where r is the ratio of the potency of the doses of Ps and E2.

$$\text{Hence} \quad \log. r = 0.1109$$

$$\text{and} \quad r = 1.291$$

$$\text{i.e.} \quad \frac{\text{potency of 150 mg. E2}}{\text{potency of 350 mg. Ps.}} = 1.291$$

$$\text{Potency of 1 g. E2} = \text{potency of 3 g. Ps.}$$

i.e. the extract E2 possesses three times the purgative activity of the same weight of the standard senna fruit from which it was made.

ACCURACY OF THE METHOD

In order to determine the accuracy obtainable by the method, doses of a laboratory standard of powdered senna leaf were given to 4 groups (1, 2, 3, 4) each of 10 mice on 9 different occasions. 2 of the 4 groups (1 and 3) each received 3 times the dose given to the other 2 groups (2 and 4). The responses of each of the 4 groups were noted. The responses of 1 high-level dose and 1 low-level dose (say groups 1 and 2) were taken to represent a potency of 100, the potency of the remaining two (groups 3 and 4) was calculated. This process was repeated by rearranging the groups so that the responses of groups 1 and 4 were taken to represent a potency of 100, and the potency of the groups 2 and 3 was calculated as before; hence, one single "assay" yielded two results. Altogether 18 such results were obtained and are recorded in Table III. The mean (M) of these 18 results was 99.44 with a standard deviation (σ) of 15.626 (or 15.714 per cent. of the mean). Limits of error ($P=0.99$) for a single assay are therefore 100 ± 40.5 per cent.

Calculation:

$$\text{Mean potency of B calculated} = \frac{1789.94}{18} = 99.44.$$

$$\text{Sum of squares of deviations from the mean} = \sum d^2 \quad 4150.832.$$

Standard deviation of a single determination

$$\sigma = \sqrt{\frac{4150.832}{18-1}} = 15.626 \quad \text{or} \quad \frac{15.626}{99.44} \times 100 = 15.714 \text{ per cent.}$$

COMPARISON OF ACCURACY WITH THAT OF OTHER METHODS

Previous workers used rats^{11, 12}, guinea pigs¹³, daphnia², etc., for the evaluation of purgative activity but none of them gave any indication of the accuracy obtainable. Munch¹ who obtained his best results with cats claimed an accuracy of only 20 to 50 per cent. Loewe³ using rhesus

TABLE III

ESTIMATION OF THE ACCURACY OF THE METHOD

Experiment No.	Group of Mice	Number of WF/kg. of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d ²
30a	A { 1 2 B { 3 4	126.3 33.5 136.6 38.1	108.93	9.49	90.0601
b	A { 1 4 B { 3 2	126.3 38.1 136.6 33.5	103.39	3.95	15.6025
31a	A { 1 2 B { 3 4	130.4 54.5 135.8 56.8	105.62	6.18	38.1924
b	A { 1 4 B { 3 2	130.4 56.8 135.8 54.5	102.29	2.85	8.1225
32a	A { 1 2 B { 3 4	173.0 82.0 187.3 43.1	89.15	10.29	105.8841
b	A { 3 2 B { 1 4	187.3 82.0 173.0 43.1	78.00	21.44	459.6736
33a	A { 1 2 B { 3 4	146.6 65.4 139.8 57.9	90.88	8.56	73.2736
b	A { 1 4 B { 3 2	146.6 57.9 139.8 65.4	100.54	1.10	1.2100
34a	A { 1 2 B { 3 4	99.2 41.7 82.7 25.0	72.91	26.53	703.8409
b	A { 1 4 B { 3 2	99.2 25.0 82.7 41.7	100.19	0.75	0.5625
35a	A { 1 2 B { 3 4	76.2 31.3 86.2 26.2	105.30	5.86	34.3396
b	A { 1 4 B { 3 2	76.2 26.2 86.2 31.3	117.52	18.08	326.8864
37a	A { 1 2 B { 3 4	119.5 42.9 109.4 53.6	100.50	1.06	1.1236
b	A { 3 2 B { 1 4	109.4 42.9 119.5 53.6	118.82	19.38	375.5844
38a	A { 1 2 B { 3 4	46.0 20.0 72.5 4.9	114.32	14.88	221.4144

BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES. PART I

Experiment No.	Group of Mice	Number of WF/kg of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d ²
b	A { 3 2 B { 1 4	72.5 20.0 46.0 4.9	61.35	38.09	1450.8481
39a	A { 1 2 B { 3 4	123.3 22.1 142.3 27.2	112.97	13.53	183.0609
b	A { 1 2 B { 3 4	123.3 27.2 142.3 22.1	107.26	7.82	61.1524
TOTALS			1789.94		4150.8320

monkey as test animal claimed an accuracy of ± 15 per cent., however there is neither data nor statement as to how many monkeys are needed to achieve this accuracy.

Geiger's original method⁵ involved the use of 72 mice per single assay; however, insufficient data is available to calculate the accuracy obtainable. Moreover, his method was improved by Grote and Woods who used 105 mice per single assay. Again, however, no figure for the degree of accuracy was given. This omission from the published methods of bioassay is a serious one, especially in view of the large number of mice used in each assay.

Collier¹⁰ states that the standard deviation of his method is usually about 20 per cent. with a slope (b) of log. dose/response line of about 80 per cent. ($b^2/s^2 = 16$). The limits of error ($P = 0.99$) of each assay using 40 mice calculated from the formula quoted by him would be 63 and 160 per cent. However, when a ratio of $b^2/s^2 = 30$ is obtained, as he states occasionally occurred, the limits of error using 40 mice would be 71 and 141 per cent. As already stated the limits of error of the method described in this paper ($P = 0.99$) are 100 ± 40.5 per cent., which on the whole is a higher accuracy than that of Collier's method.

APPLICATIONS OF THE METHOD

The method was found very satisfactory when used to assay senna leaf, senna fruit and extracts and commercial preparations made from these drugs, also the pure glycosides, sennosides A and B, and the pure anthracene compounds, aloe-emodin and aloe-emodin anthranol. The method was further applied to cascara, rhubarb and to preparations of these drugs also with satisfactory results, although in some instances slight modification is necessary. It is hoped to publish details of these investigations later.

The results of many of these assays are incorporated in the paper of Dr. J. W. Fairbairn¹⁴.

SUMMARY

1. A method for the biological assay of vegetable purgatives based on the number of wet faeces produced by groups of mice after dosing is described.

2. The relation of the number of wet faeces per kg. of mouse to the logarithm of the dose was found to be linear.

3. 40 mice divided equally into 4 groups were used in each assay. 2 groups received the standard preparation and the other 2 groups received the test preparation. The standard deviation of a single determination based on 9 such assays was estimated to be 15.7 per cent. The limits of error ($P=0.99$) for a single assay are 100 ± 40.5 per cent.

4. A special cage has been designed for this assay, and it has been found advantageous to incorporate a definite proportion of water in the diet, during test.

5. The method described is not only convenient in use but also gives a comparatively high degree of accuracy.

6. The method has been successfully applied to senna leaf, senna fruit and extracts and commercial preparations made from these drugs, pure glycosides (sennosides A and B), and pure anthracene compounds (aloe-emodin and aloe-emodin anthranol).

ACKNOWLEDGEMENTS

The author wishes to thank the Department of Pharmacology for providing facilities for keeping the mice and especially Dr. F. J. Dyer, under whose supervision the preliminary part of this work was carried out; Dr. J. W. Fairbairn, the Head of the Department of Pharmacognosy, for encouragement during the course of this work, and for help in preparing the manuscript; and Mr. A. H. Fenton, B.Pharm., for carrying out a trial on the method suggested by Geiger⁵.

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THE ACTIVE CONSTITUENTS OF THE VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART I. GLYCOSIDES AND AGLYCONES

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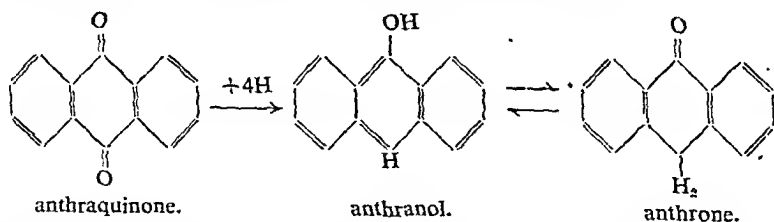
THE following are the common vegetable drugs containing anthracene derivatives; Senna, leaf and pod (*Cassia acutifolia* and *C. angustifolia*); Cassia pulp (*Cassia fistula*); Cascara sagrada (*Rhamnus purshiana*); Frangula (*R. frangula*); Rhubarb (*Rheum* spp.) and Aloes (*Aloe* spp.). These drugs act as irritant purgatives and all respond to the Bornträger test¹ or suitable modifications^{2,3} by means of which the anthracene derivatives are converted into free anthraquinone compounds which give pink to red colours in alkaline solution.

ANTHRACENE DERIVATIVES

The anthracene derivatives occur either free or in the form of glycosides, usually with glucose, though glucofrangulin also contains the sugar rhamnose⁴. The following aglycones have been reported:—

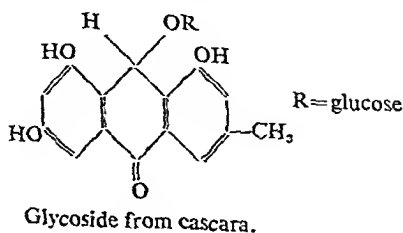
(1) *Anthraquinone compounds*, e.g. emodin, aloë-emodin, rhein. These compounds occur in all the drugs mentioned above, sometimes in very small amounts as in aloes⁴ and in senna leaf⁵ and sometimes in quite large amounts as in cascara⁶ and rhubarb.⁷

(2) *Anthranol compounds*. Anthranol (and its tautomeric isomer anthrone) is a reduced form of anthraquinone as shown



Hydroxy derivatives of anthranol and anthrone, corresponding to those of anthraquinone, occur frequently in these purgative drugs, e.g. aloë emodin anthranol in aloes.⁸

(3) *Oxanthrone compounds*. These are intermediate between anthraquinones and anthranols. Schindler⁹ has shown that cascara bark contains a glycoside based on this structure:—



On hydrolysis the aglycone is rapidly oxidised to emodin.

(4) *Dianthranol compounds*. Stoll, *et al.*¹⁰ suggest that the aglycones in senna leaf may be dianthranol or dianthrone compounds which on oxidation in alkaline solution give a rhein-like compound.

For the purposes of this preliminary investigation these anthracene derivatives are classified as (a) glycosides, (b) free anthranol compounds, and (c) free anthraquinone compounds.

ACTIVE CONSTITUENTS

Since all these anthracene purgative drugs can be made to give the Bornträger reaction under suitable conditions, it was natural that attempts should be made to determine the total content of anthracene derivatives as anthraquinones (calculated from the intensity of the red colour) and see if this amount corresponded to the biological activity of the pure anthraquinones prepared synthetically or otherwise. However, it soon became apparent that the "total anthraquinone content" could not account for the activity of these drugs. Thus Tutin and Clewer¹¹ found that 100 mg. of aloe emodin, or of emodin or of rhein were practically ineffective on human beings. This quantity of anthraquinones correspond to 4 g. of cascara bark, which is the maximum dose (B.P. 1932). Similarly, a recent attempt to correlate the colorimetric assay of frangula extract with its biological assay led the author to conclude that no parallelism exists between the two types of assay¹². Similar conclusions were arrived at by Astruc and Giroux for cascara¹³, and Ström and Kihlström for rhubarb⁷.

An interesting series of experiments by Green, King, Beal *et al.* on cascara extract^{14,15} seemed to offer an explanation of the superior activity of the crude drug and its preparation over the pure anthraquinones. They showed a definite synergistic action when the anthraquinones, aloe emodin, emodin and chrysophanol were given together to guinea-pigs. The response was much greater than with similar doses of these compounds separately, and as these compounds were stated to exist together in the drug extract it seemed reasonable to suppose that synergism of the anthraquinones accounted for the purgative action of cascara.

On the other hand Casparis and Maeder¹, working on the similar drug frangula bark, concluded that the total activity of the bark was due to the glycoside gluco-frangulin. In fact they found (by experiments on man) that this glycoside was much more active than the corresponding amount of bark; the loss of activity, when in the crude drug, they attributed to the tannins present in the bark.

Straub and Gebhardt¹⁶, working on senna leaf, discovered two active glycosides whose activity appeared to account for a large proportion of the activity of the leaf. Their work was continued by Stoll, *et al.*¹⁰ who isolated the glycosides in crystalline form and called them sennoside A and sennoside B.

Thus these recent series of experiments suggest that the activity of the anthracene purgatives may be accounted for by either (a) synergism of the free anthraquinone compounds as in cascara, or (b) highly active glycosides as in senna leaf and frangula bark. It is interesting to note that in both senna leaf⁵ and frangula bark¹⁷, more than nine-tenths of the anthracene

derivatives present occur as glycosides. Hence a determination of "total anthraquinones" would be virtually a determination of the glycosidal content, which (as stated above) is said to account for the total activity of these drugs. However, I have already quoted papers to show that such a correlation has not been found for every member of the group.

I decided therefore to investigate the whole series of anthracene drugs to see if any generalisation could be made as to what are the active constituents. The remainder of this paper describes the preliminary work towards this end, viz., the determination of the relative activity of (a) glycosides, (b) free anthranols and (c) free anthraquinones. The results of the experiments recorded show that for senna leaf, senna pod, sennosides A and B, rhubarb and cascara, the anthracene derivatives are highly active in the glycosidal form; less active as free anthranols and much less active as free anthraquinones. A discussion of these findings is given at the end of the paper.

EXPERIMENTAL

Chemical and Biological Assays. Necessary requirements for this type of investigation, are reliable methods of chemical and biological assay. The *chemical assays* were based on the colorimetric methods of Kussmaul and Becker for senna⁵, and Björling and Ehrlén for frangula¹². Various modifications were necessary and it is hoped to publish details of chemical assays for each drug later. The glycosidal content of senna and preparations were estimated as sennosides A + B; that of cascara and of rhubarb as the oxidised aglycone, emodin. In order to make the figures comparable the glycoside content of senna is also given in terms of aglycone A + B. The *biological assays* were carried out by Mr. T. C. Lou¹⁸.

1. *Preliminary experiments on Senna leaf.* Portions of powdered Tinnevely leaf were subjected to increasing degrees of hydrolysis and the purgative activity of these fractions and that of the original leaf were compared by a bio-assay method based on that of Geiger¹⁹. The results, recorded in Table I, indicate that mild hydrolysis has little effect on the purgative activity but that more vigorous hydrolysis, and

TABLE I
PURGATIVE ACTIVITY OF SENNA LEAF FRACTIONS AFTER VARYING DEGREES OF HYDROLYSIS

Treatment	Temperature and Time	Bio-assay
1 Untreated leaf	—	100
2 Warmed with 0.16N hydrochloric acid in atmosphere of nitrogen	20° C for 18 hours 70° C for $\frac{1}{2}$ hour	88
3 Warmed with 0.1N hydrochloric acid in atmosphere of carbon dioxide	90° C for $\frac{1}{2}$ hour 20° C for 22 hours	77
4 Boiled in water under reflux	100° C for 2 hours	36
5 Boiled in 1.5N hydrochloric acid under reflux	approx 120° C for 2 hours	0

possibly oxidation (produced either by boiling in water or warming in strong acid solution in air) led to a marked loss in activity.

2. *Quantitative experiments using senna leaf glycosides.* The preliminary experiments suggest that hydrolysis of the leaf constituents, and possibly oxidation, leads to loss of activity. I obtained the leaf glycosides sennosides A and B in pure form and decided to repeat the previous experiments on a more quantitative basis.

A solution of sennoside A of suitable concentration was divided into three portions. One portion was hydrolysed and the liberated aglycones extracted and purified (as in the chemical assay process⁵); these were administered to mice in suitable doses. The second portion of the sennoside A solution was hydrolysed and oxidised (as in the chemical assay process⁵) and similar doses of the purified products were given to mice. The third portion of the original solution was used as a control.

The results, recorded in Table II, show that the aglycone possesses about 1/3 of the activity of the parent glycoside, whereas the oxidised aglycone possesses no activity at all (when given in similar doses).

TABLE II
BIOLOGICAL AND CHEMICAL ASSAYS OF SENNOSIDE A AND FRACTIONS

Material	Bio-assay	Chemical assay
1. Sennoside A	100	100
2. Hydrolysed Sennoside A (3 N sulphuric acid at 95° C. for ½ hour)	32.5	96.4
3. Hydrolysed and oxidised sennoside A (above, oxidised in N sodium hydroxide and hydrogen peroxide)	0	85.3

Note: In theory, the results for the chemical assays should all be 100. The discrepancy in Expt. 2 is within experimental error; that in Expt. 3 can be accounted for by having to heat with hydrogen peroxide longer than usual, owing to the high concentration of the aglycones.

3. *Quantitative experiments using senna pod.* Similar experiments to those done on senna leaf glycosides were repeated on senna pod which is also said to contain sennosides A and B.²⁰ A large sample of Alexandrian senna pod was thoroughly mixed and stored in a cool place; a few hundred g. reduced to No. 60 powder was used as standard (= P_g). A potent extract of some of this powder was made by evaporating an infusion* under reduced pressure to a solid extract (E_g). This extract was assayed biologically and chemically against the standard powder. Some of it was then hydrolysed by heating in 5 per cent. hydrochloric acid at 90°C. for ½ hour, cooled and neutralised with sodium hydroxide. This hydrolysed product was re-assayed chemically and biologically against the Standard powder to which a calculated quantity of sodium chloride was added to balance that formed during the neutralising of the hydrolysate. The results of these experiments are shown in Table III.

These experiments on senna pod confirm the conclusions derived from the experiments on senna leaf and glycosides, in that hydrolysis leads to a marked loss in activity. They also show that a determination of the anthracene derivatives as "total anthraquinones" would be no guide

* Chemical assays showed that all the anthracene derivatives passed into an infusion made under suitable conditions.

VEGETABLE PURGATIVES—PART 1

to the biological activity. Thus in experiments 2 and 3:—biological activity is in ratio 6·8 : 1; glycosidal content is in ratio 8·3 : 1; "Total anthraquinones" is in ratio 1·1 : 1.

TABLE III

BIOLOGICAL AND CHEMICAL ASSAYS OF SENNA POD AND FRACTIONS

Material	Bio-assay	Chemical Assay (Results expressed as mg./g.)			
		Glycosides		Free	Total
		Sennosides A+B	As Aglycones	As Aglycones A+B	As Aglycones A+B
1. Standard senna pod powder P _s	100	32·0	20·0	2·0	22·0
2. Potent extract of Pod E ₁ ...	(i) 301 (ii) 376 } 339	132·4	82·8	8·2	91·1
3. Hydrolysed extract ...	Approx. 50	16·0	10·0	71·5	81·5

4. *Quantitative experiments using Rhubarb.* The experiments already described were made on senna and its preparations, where the aglycones were "artificially" produced by hydrolysis. In rhubarb and cascara, however, there is present "naturally" a large proportion of free aglycones, so that experiments carried out on these drugs will not only decide whether what is true of senna is applicable to other anthracene purgatives; but the proof of relative activity of the glycosides and aglycones can be obtained without resort to the drastic process of acid hydrolysis. Crude drugs and their preparations are complex mixtures and it is not always possible to forecast what effect hydrolysis of other constituents will have on the biological activity.

A weighed quantity of a Standard sample of powdered Chinese rhubarb (R_s) was exhausted with ether and acetone to remove the free emodins and the exhausted material was dried and re-weighed. There was a loss in weight of 15 per cent. The activity of this exhausted material (R_s) and of the Standard powder were compared by the biological and chemical methods already mentioned and the results are shown in Table IV. In calculating the potencies of R_{ex} allowance was made for the 15 per cent. loss in weight on exhaustion.

TABLE IV

BIOLOGICAL AND CHEMICAL ASSAYS OF RHUBARB AND FRACTIONS

Material	Bio-assay	Chemical Assay Total Anthracenes as Emodin : mg./g.
1. Standard rhubarb powder R _s ...	100	30·5
2. R _s exhausted with ether, etc. = R _{ex} ...	(i) 106 (ii) 84 (iii) 107 } = 99*	7·2*

* Allowance made for loss in weight of 15 per cent. on exhaustion.

These results fall into line with those already obtained. The removal of a large amount of free aglycones did not result in any loss of activity, thus indicating that the main activity lies in the (ether-insoluble) glycosides.

aglycone. The glycoside is not hydrolysed in the stomach and so reaches the large intestine where it is hydrolysed and the liberated (and presumably oxidised) aglycone then exerts its action. The work described in this paper has not only confirmed this theory for senna leaf and shown that the same is true of senna pod, rhubarb and cascara, but also indicates that the sugar moiety plays the further role of "protector," preventing the orally active anthranol from oxidation during storage to orally inactive anthraquinone. In all the drugs studied it was found that nearly all the free aglycones are present in the anthraquinone form (though the glycosides contained "anthranol" aglycones), indicating that after hydrolysis the liberated anthranols are fairly rapidly oxidised during storage. Furthermore, if the pure senna glycosides are heated in N sodium hydroxide with hydrogen peroxide, no oxidation takes place after hydrolysis of the glycosides (in acid), however, the aglycones are rapidly oxidised under similar conditions. This "protector" theory is in line with what is known of the constitution of these glycosides (e.g. in cascara⁹, aloin²⁹). The sugar is attached to the meso group and so would protect the anthranol structure.

These theories appear to conflict with that of Liddell, King and Beal¹⁴, who claim synergism of the anthraquinones as the explanation of the activity of cascara. While their experiments do show that synergism occurs, they fail to show that this synergistic effect accounts for the total activity of the crude drug. They used 1.5 ml. of a commercial sample of fluid extract of cascara (U.S.P.) as standard in the bio-assay, but unfortunately did not determine the amount of anthraquinones in this standard. Gibson and Schwarting⁶ have shown that this amount varies considerably; their highest figure is 2.9 mg. per ml. Thus, at the most 1.5 ml. of standard may contain about 4.5 mg. of anthraquinones, whereas Liddell *et al* found it required 12.5 to 25 mg. of the synergistic mixture to produce a similar effect. However, if sufficiently large doses of free anthraquinones are given by the mouth, purgation results; indicating that a proportion has reached the large intestine. In such circumstances, synergism may be an important factor. To be effective, comparatively large quantities must be given, e.g. 100 to 300 mg. for man^{11,27}. This quantity corresponds with the dose of the synthetic anthraquinone, 1 : 8 dihydroxyanthraquinone* (Istin) of which 150 to 450 mg. is necessary as a purgative.

The results also show that a chemical assay which merely determines the total content of anthracene derivatives, irrespective of their form of occurrence, will not correspond to the purgative activity. An interesting example of this occurs in a paper recently published from Finland on the chemical and biological assays of Chinese and rhapontic rhubarbs⁷. The former contains less "total anthraquinones" yet is twice as active as the rhapontic and the authors conclude that the chemical assay does not give a true picture of the laxative effect. However, their figures for "combined anthraquinones" (glycosides) are in the ratio of 9.25 to 5.08 respectively, which corresponds very closely to the biological assays.

FURTHER WORK

Having established that the glycosidal fraction of these crude drugs possesses the main activity, I propose to study this fraction in greater detail, as it is obvious that the aglycones of the various glycosides vary considerably in their activity, e.g. the aglycones of sennosides A and B are much more active than the simple anthranol of aloe emodin. It is further hoped to publish details of chemical assays and to examine galenicals made from these drugs to see whether all the glycosides are extracted and whether they are preserved during storage. Hazleton and Talbert³⁰ state that the U.S.P. fluid extract contains only 35 per cent. of the activity of senna leaf. Preliminary experiments which we have performed on a commercial sample of dry extract of cascara showed it was slightly less active than the crude drug; whereas it should be about four times as active. Similar experiments on a dried extract of senna pod made by the B.P. method showed that it had only 1/6 of the activity of the pod, instead of 3 to 4 times its activity.

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1. A review of the published theories which seek to account for the activity of purgative drugs containing anthracene derivatives is given.

2. The relative activity of anthracene derivatives occurring as (a) glycosides, (b) anthranols and (c) anthraquinones, in certain crude drugs has been determined by biological means.

3. A similar investigation of isolated glycosides, anthranols and anthraquinones has been carried out.

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DISCUSSION

The Papers on Vegetable Purgatives by Mr. Lou and Dr. Fairbairn were discussed together

THE CHAIRMAN said that until recently there had not been a really satisfactory assay of these drugs. Mr. Lou's method was a development of that described last year by Collier and Harris.

DR. I. MICHAELS (London) said that senna pods were said to be more certain than the leaves in their laxative action, and to cause less griping. It appeared that sennosides A and B were present in both, and it would be logical to conclude that the pods contained a higher proportion of active principles than the leaves. It would be interesting to know the comparative figures for the active principles of Alexandrian and Tinnevely pods so as to be able to assess the reason for the market price of the Alexandrian pods being three times that of the Tinnevely pods. Griping was said to be associated with the anthracene compounds. The seeds were said to be the cause of griping, and yet it was recorded that the seeds did not contain anthracene derivatives. Modern theory stated that the senna glycosides passed unchanged through the stomach to the intestines, where they were absorbed into the blood stream: here they were hydrolysed. There was a latent period of 10 to 12 hours before the active emodin reached the large intestine, where it stimulated the

peristaltic movements of purgation. The emodins in a badly prepared extract were responsible for the griping pains by stimulating peristalsis in the small intestine. Mr. Lou's method complied with the accepted principles of biological assay. There could be little disagreement with the choice of powdered senna as a standard for the assay of senna and its preparations, although there was room for controversy as to the method of administration. It should be regarded, however, as an intermediate stage in the establishment of a standard based on a compound of known composition.

DR. E. F. HERSANT (Dagenham) said if the glycoside were not hydrolysed in the stomach, was it not possible that after hydrolysis in the large intestine the unoxidised aglycone would partly account for the greater activity of the glycosides.

DR. F. SAID (Egypt) said that glycosides were usually hydrolysed by acids, and if they were not hydrolysed in the stomach they were unlikely to be hydrolysed by the alkali in the duodenum. If the glycosides were absorbed in the stomach and were excreted in the intestine, would it be possible to inject a solution of an anthraquinone and see if it was so excreted.

MR. V. REED (London) asked if the author had any explanation of why the public taste for senna preparations had altered so much in the last 30 or 40 years. At first there had been a big demand for senna leaves, then senna pods had come in. Now there were senna leaves, Tinnevely senna pods and Alexandrian senna pods, all taken for the same purpose. Was there any real difference in the actual active principle of the three things?

DR. T. E. WALLIS (London) said he was glad to see a Department of Pharmacognosy taking an interest in the biological side, as was the case in similar departments in other countries. He asked for Mr. Lou's opinion on the use of *Daphnia* in this type of work.

DR. D. C. GARRATT (Nottingham) said that it was necessary to get an accurate chemical assay before they could fully appreciate the really good work that had obviously been done.

DR. WALKER (London) said that in the last twelve months he had had to make comparisons clinically of liquid extracts of senna and frangula. The experience had been that many of the senna extracts examined had shown very poor activity compared with senna pods themselves, whereas the difference in the activity in frangula extracts had not been so marked. It would appear that the conclusions reached had been on single samples of powdered senna and cascara. Had the author gone far enough yet to offer any information as to variations between different samples of the powdered drugs.

DR. F. HARTLEY (London) asked whether, in arriving at the conditions for the hydrolysis of glycosides in Table 1 the times and conditions had been arbitrarily chosen, or were they known to be the most favourable for hydrolysis? If Dr. Fairbairn could give supplementary data on the rate of hydrolysis of the glycosides, it would throw light on the

conditions. It was said that by boiling at 110° under a reflux with water for two hours, 60 per cent. of the senna glycosides had been hydrolysed. Could Dr. Fairbairn say whether substantially all that decomposition had occurred during a shorter period such as 15 or 20 minutes.

MR. H. DEANE (Long Melford) said that the method appeared to be accurate for testing purgative drugs. Human volunteers had so far seemed the only way of testing, but the staff of laboratories considered that it was not part of their duties. Senna was more sensitive to heat than rhubarb or cascara; a freshly-made infusion of senna pods was much more active than a liquid extract made from the same pods.

MR. H. B. MACKIE (Brighton) said that it had long been known that with cascara partial hydrolysis decreased therapeutic activity, but increased palatability. With a solid preparation which could be protected by coating palatability was unimportant. In liquid preparations, the therapeutic action had to be balanced with flavour and other factors, if the product was bitter and nauseating it could not be swallowed. In the preparation of decoction of aloes, the aloes was boiled with potassium carbonate and the dose of decoction was equivalent to twice the dose of aloes to get the same therapeutic result, but it was then pleasant to take; 50 per cent. of the activity of the aloes had been lost in boiling.

DR. FAIRBAIRN, replying, said that the glycosides were the active principles, but the particular glycosides present in different drugs might vary, thus giving different activities. Regarding anthranols, presumably as soon as the glycosides were hydrolysed the free anthranol enhanced the activity. It was true that the stomach contained acid, but Table I showed that it needed fairly strong acids at high temperature to produce hydrolysis. Dr. Collier had reported last year that he had injected liquid extract of senna into the veins of mice, but there had been no purgative action.

MR. LOU said it was, generally speaking, much better if a definite compound was used as a standard, but unfortunately these purgative drugs contained various glycosides which might be different in structure. If sennoside A or B was adopted as a standard, and the product assayed on either compound, the result might be in conflict with a chemical test. It was obvious that workers in this field ought to be careful about the element of biological variation. Statistical considerations had been applied to all the tests they had made and the errors were within 15 per cent. They had no experience with the *Daphnia* method. There was a wide zoological gap between *Daphnia* and human beings, and they had thought it preferable to use animals where the gap was much smaller. He had tested a few preparations on himself. Those preparations which were inactive in himself, or had very low potency, had no activity in mice. In Munch's book on bio-assays, there were data to show that the relation of dose between a mouse and a human being differed with different purgatives. For senna the ratio was about 1 to 300. For instance, if the minimum effective dose for mice was 6 mg. the human dose would be 1.8 g. which was within the dose range of the B.P. for senna. He had carried out laboratory experiments on extracting both pods and leaves, but the latter gave difficulty because of the large quantity of mucilage they contained.

NOTES ON MICROBIOLOGICAL ASSAYS USING *LACTOBACILLUS LACTIS DORNER*

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The announcement by Shorb^{1,2,3} that *Lactobacillus lactis* Dorner responded to a factor present in liver extracts to an extent roughly proportional to their clinical value, opened up the possibility of a microbiological assay of anti-pernicious anæmia preparations. The isolation of the growth factor by Rickes *et al*⁴, who named it vitamin B₁₂, and the demonstration by West⁵ of the anti-pernicious anæmia potency of Vitamin B₁₂, strengthens this possibility.

Most types of bacteria moulds, etc., are able to synthesise vitamin B₁₂ but several organisms have been found which lack this property and these have been tried for assay purposes. The present communication deals with a method of assay in liquid culture using *L. lactis* Dorner.

Shorb^{2,3,6} and Shive⁷ have drawn attention to a marked tendency for this organism to behave in an inconstant manner under certain conditions. This uncertainty has been confirmed by the unpublished experiences of many workers in this field and, for many months, investigations in these laboratories were hampered by erratic assay results. The technique finally evolved and described below seems to be free from the disabilities reported by other workers.

The principles utilised in the final method are identical with those influencing all other microbiological assays (Shaw⁸). The test inoculum must be minimal. The organisms in the test inoculum must be young, in a highly active phase of growth, and to have been maintained on a complete medium. The medium must be free from other growth factors. The difference between *L. lactis* Dorner and other organisms is that the former is much more prone to variation formation. It was found that when optimum conditions had been established, the assays were more consistent than with any other microbiological assay performed in these laboratories.

The basal medium employed consists of sulphuric acid-hydrolysed casein with added tryptophane, the usual members of the vitamin B complex group and tween 80. T.J. factor, another essential supplement, is supplied as a concentrate of potato extract consisting of the ether precipitate from a phenol extract of the fraction insoluble in alcohol (80 per cent.) but soluble in alcohol (50 per cent.). One advantage of this source of T.J. factor is that unlike with enzyme digested casein used by Shive,⁷ or with tomato juice, used by Shorb,^{1,2,3,6} it is possible to use a large excess of T.J. factor without at the same time adding *L. lactis* Dorner factor. Another advantage is that since the current batch of concentrate is used at a final dilution of 1/50,000, it is possible to use the same batch of potato extract over a considerable period. All batches of tween 80 are not equally suitable. One sample which did not give a clear 10 per cent. solution in water was useless.

The reaction of the final medium is adjusted to pH 5.5 and the glucose, potato extract and preparation to be tested are added aseptically to the previously autoclaved basal medium. If the glucose is added to the medium prior to autoclaving, the resultant broth may support the growth of *L. lactis* Dorner in the absence of liver extract or other vitamin B₁₂ containing material. Under these circumstances the "blank" is high and may even approach maximum turbidity. Autoclave treatment at pH 7 to 8 is more likely to upset the assay than when the pH is approaching 5.5 at which pH the phenomenon is rarely observed. One does not always obtain this growth potentiation on autoclaving and one possible explanation of failure to do so may be that some samples of glucose solution may become acid on autoclaving. If the pH is lowered before the reaction between glucose and media constituents can occur, it would be equivalent to autoclaving at a low safe pH.

The strain used was isolated as a single colony from soya bean medium and has been maintained for upwards of 150 daily sub-cultures on peptone agar, fortified with proteolysed whole liver, tween 80 and potato extract. Its behaviour has remained constant throughout this series of cultivation.

For assay purposes an overnight agar slope culture is washed into 10 ml. of basal medium fortified with proteolysed whole liver, and this allowed to incubate for 5 to 6 hours at a temperature of 37°C. The broth culture is centrifuged, the sedimented organism washed 2 or 3 times with basal medium deficient in vitamin B₁₂ and finally suspended to an opacity of approximately 1/5th, in either vitamin free basal medium or saline, immediately before use. Three drops of this suspension are used to inoculate 10 ml. of test medium.

Growth is usually satisfactory after 16 to 18 hours incubation, but, if the inoculum is too small or the organism is not in good condition, incubation up to 48 hours may be necessary. Exposure of the washed organism to saline solution for more than about 1½ hours may convert the organism from a plump bacillus, staining solidly Gram-positive, into a thin one with the body of the organism Gram-negative showing Gram-positive beads simulating the appearance of a chain of streptococci. Further exposure to saline solution might even result in a completely Gram-negative filamentous organism from which it is difficult to get a satisfactory culture. In contrast to the report of Shorb³ we have not found that the length of incubation affects the assay values. The general levels of growth are slightly higher but the test solution is affected equally with the control. Probably as a consequence of the aseptic addition of glucose, the test medium is water white, and during the period when glucose was autoclaved with the medium it was found that, in general, a low blank was obtained when the colour of the medium was minimal, the colour being presumably an index of glucose decomposition.

To ensure regular results, it is essential to use constant-bore test tubes in order to have a constant surface-area/volume ratio. Incubation is best performed in a large room-incubator and the test tubes must not be shaken during the growth period. Sterility of the glucose is ensured by

the candle filtration of a 40 per cent. solution. Liver extracts in ampoules are assumed to be sterile, otherwise dilutions are made from preparations which have been allowed to stand in the presence of 1 per cent. phenol. The final dilution of the liver extract in the final medium is usually so great as to eliminate the effect of any phenol carried over. Where necessary, the preparation to be assayed is autoclaved and this does not appear to have any deleterious action. It is however best to avoid autoclaving the test sample.

It has been found convenient to make the basal medium up to 9/10ths final strength, to distribute this into aluminium capped test tubes in 9 ml. quantities and then to autoclave these tubes in lots of about 500 tubes. The tubes are prepared for use by adding the requisite amounts of glucose, potato extract and vitamin B₁₂ preparation all in a volume of 1 ml. The autoclaved tubes may be used at any time up to 14 days after autoclaving without affecting the assay in any way; thus enabling a large number of assays to be conducted with medium is known to be identical in every respect.

The optimum conditions for autoclaving are obtained by the use of a large autoclave in which the tubes are placed in special racks so arranged that there is 1 in. free space around each tube. The autoclave is fitted with a recording thermometer and so it is possible to heat up during 10 minutes, to maintain at a temperature of 115°C. for 10 minutes and to take the same period to cool down. There is also available a permanent record of the autoclave history of that batch of tubes.

According to Shorb⁶, *L. lactis* Dorner maintained on yeast-extract-agar with or without tomato juice, gives irregular results, and growth may be inhibited by some liver extracts. The strain used in these laboratories with the described technique gives very reproducible results and the dose-response curve for all liver extracts assayed, for pure vitamin B₁₂ and thymidine, have been found to be identical whether assayed individually or in mixture. Thymidine has only 1/10,000th of the activity of vitamin B₁₂. Values obtained from the assay of mixtures are invariably the sum of two components. Inhibitors might be expected to affect the shape of the growth response curve and the values obtained on blending.

Growth-response curves are established for each assay by using 5 levels of the standard preparation. It is desirable to put up an equal number of levels of unknown, endeavouring to arrange the dilutions so that the reading of each level of the test coincide with the corresponding levels in the control series. For accurate work it is desirable to use 6 tubes at each level and to repeat the assay at least 3 times. Ordinarily 3 tubes at each of 3 or 4 levels provide a fairly accurate estimation. A typical growth response curve is shown in Figure 1.

Lester Smith and Cuthbertson⁹ reported the presence of four substances with growth activity for *L. lactis* Dorner which may be present in liver extracts. These substances have different migration velocities on paper strip chromatograms when developed with aqueous butyl alcohol. Two of the substances, both with anti-pernicious anæmia activity, migrate less rapidly than riboflavine, and two, with no anti-pernicious anæmia

potency, migrate more rapidly than riboflavine, one being thymidine, which is found very near to the advance front. The possibility of other *L. lactis* Dorner-active materials being present in a liver extract in addition to vitamin B₁₂ must therefore be taken into consideration. This possibility has been dealt with by subjecting various extracts to paper strip chromatograph (Shaw¹⁰). A spot of extract or other preparation (1/200th ml. of an extract assaying at 10 µg/ml. is satisfactory) is placed on a 1 cm. strip of Whatman No. 1 filter paper and, after drying, this strip is suspended in normal butyl alcohol, saturated with water, so that the butyl alcohol level is about 2 cm. below the applied spot. The butyl

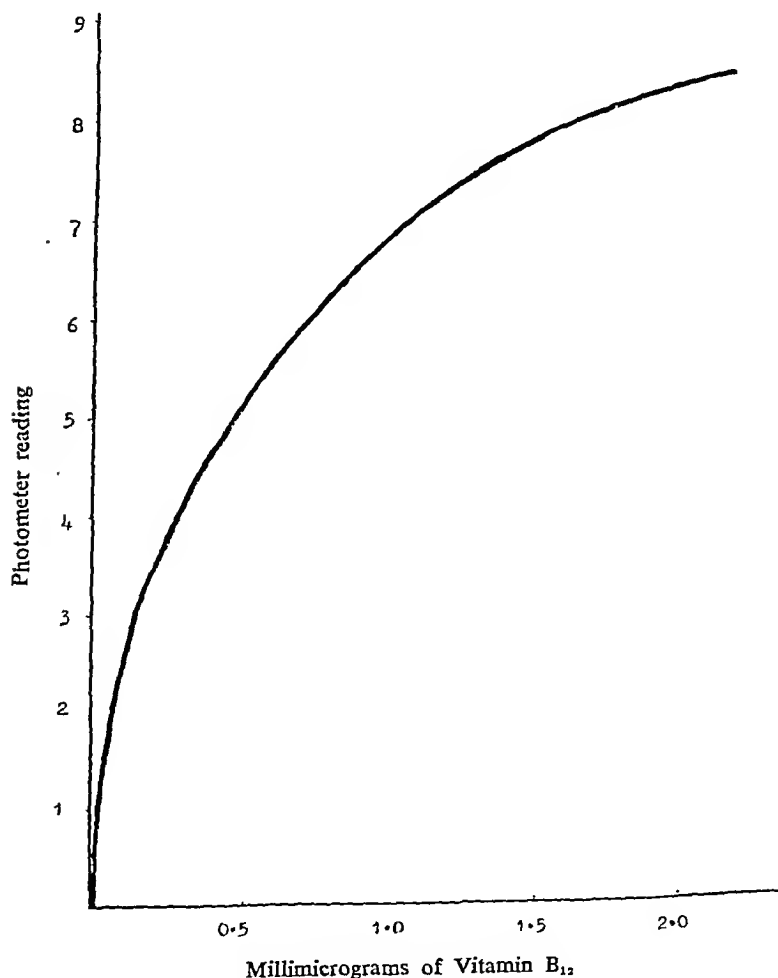


FIG. 1.—Growth-response curve vitamin B₁₂, *Lactobacillus lactis* Dorner. Average of 25 tests. 10 ml. tubes—1 cm. cell.

alcohol is contained in the bottom of a glass cylinder and the sides of the cylinder extend to about 13 cm. above the spot. The whole is pro-

from light and the butyl alcohol travels up the strip, evaporates at the top of the cylinder and deposits all of the rapidly migrating material at this point of the strip at the advanced front.

A small amount of riboflavine may be placed on the same spot as the liver extract and after about 48 hours development, it will be found that the advance front is 13 cm. from the spot and a riboflavine belt (identified by examination under ultra violet light) is found about 6 cm. from the spot. The strip is cut at this point, each portion extracted with water and the extract assayed, thus giving the amount of activity which migrates both less and more rapidly than riboflavine.

It was found that the speed of migration of the main *L. lactis* Dorner-active fraction was so much slower than that of the other fractions that latterly the strip has been allowed to develop for a longer period, i.e., 14 to 21 days, when it has been found that most of the activity has left the spot, accumulating in a peak about 2½ cm. from the spot. There is then a considerable length of filter paper which is devoid of activity and the rapidly migrating growth materials accumulate at the advance front. The strip is examined by cutting it up into 0.5 cm. sections, extracting each with water and assaying separately. A typical liver extract gives results as shown in the histogram in Figure 2.

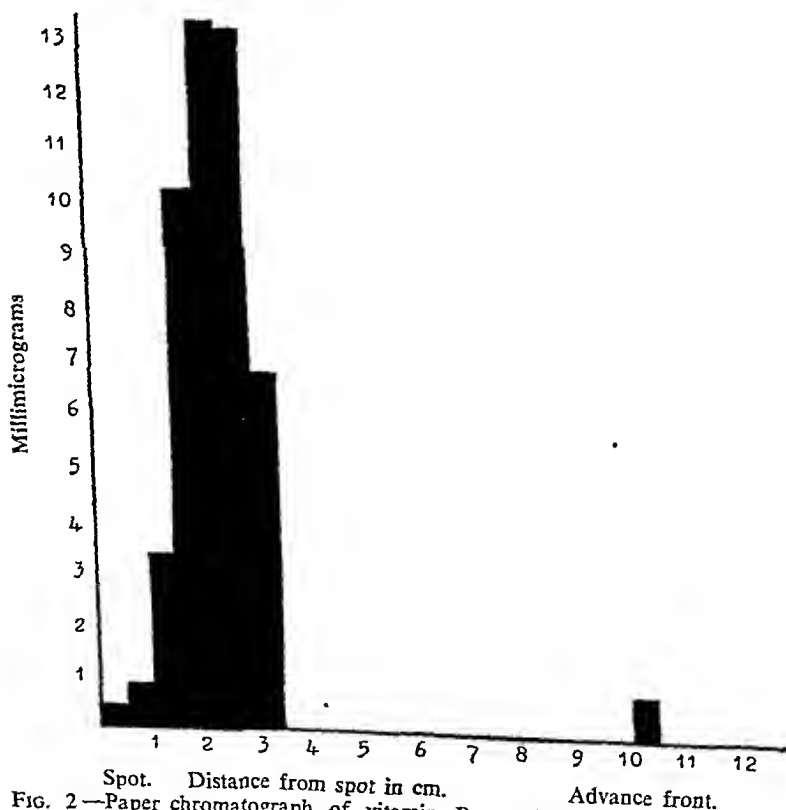


FIG. 2—Paper chromatograph of vitamin B₁₂ in liver extract Product "A," 0.005 ml. developed for 18 days

A direct comparison has not been made with the anti-pernicious anæmia factor of Lester Smith³. Dr. Lester Smith has stated that there might be a slight difference between his preparation and that of Dr. Rickes.

From Table I it will be seen that proteolysed whole liver assays at 18 µg of vitamin B₁₂ per g., and that oral liquid liver extracts range from 18 µg. to 7.5 µg. of vitamin B₁₂ per ml. It will also be noticed that one oral liver extract, which is stated to have been proteolysed, has little or no activity. Samples of parenteral products prepared by this manufacturer are also exceptionally low in *L. lactis* Dorner factor. From assays performed on comparatively small samples of liver, which had been subject to extensive laboratory treatment with papain, it would seem that proteolysed whole liver contains the entire potency of the liver used. Oral liver extracts in general are but lightly fractionated, and the final potency is probably a function of the extraction efficiency and the degree of accidental natural autolysis.

The vitamin B₁₂ content of high potency refined parenteral extracts was found mainly to fall within the range of 7.5 to 16 µg. of vitamin B₁₂ per ml. There are two exceptions, one of American and one of Continental manufacture, which assay at 30 µg. of vitamin B₁₂ per ml. In a similar manner the crude parenteral liver extracts fall in a group containing 2 to 4.5 µg. of vitamin B₁₂ per ml. A disturbing discrepancy was found when different batches of the same brand of high-potency parenteral liver extract were examined. Six batches from manufacturer "E" ranged from 0.3 to 16 µg./ml., three batches from manufacturer "B" varied from 2.5 to 13 µg./ml. These discrepancies were confirmed by repeated assays, taking care to assay the different batches of liver extract on the same day with the same batch of assay medium and against the same standard. In view of the large batch discrepancy shown, a search was made for the possible presence of inhibitory substances in the low-potency extracts. Chromatographic spectra for these extracts were normal with no unexpected increase in potency at any point. If there had been any inhibitory substance present one would not expect this "unknown" to migrate at the same speed as the *L. lactis* Dorner factor. Confirmatory evidence was obtained for the absence of inhibitors by blending low-potency extracts with a solution of crystalline vitamin B₁₂, and assaying the mixture. Values were obtained equal to the sum of the two components assayed separately. A difference would have been expected in the presence of an inhibitor unless the amount of inhibitor was just sufficient to inhibit the activity present in the liver extract alone.

Chromatographic analyses also provide a means of assessing the significance of other growth factor for *L. lactis* Dorner in liver extracts. By upward development there was in some preparations a small amount of activity present in the advance front after 14 days development. This is presumably due to thymidine, but in no case did it amount to more than 5 per cent. of the total Dorner activity. Other batches, although with similar spectra, showed no activity at the advance front. Proteolysed

GROWTH ACTIVITY OF LIVER EXTRACTS

whole liver, oral liver extracts, highly refined and crude parenteral liver extracts were similar. Downward development in the few samples examined gave entirely comparable spectra except that, since the advance front had proceeded beyond the limits of the strip, no advance front was present. In no case was there any evidence of a significant secondary peak. An illustrative histogram is shown in Figure 1. The absence of subsidiary peaks does not necessarily mean that the four *L. lactis* Dorner active substances described by Lester Smith and Cuthbertson⁴ are absent, but provide evidence that they are not present in sufficient quantities to effect the assay.

As a consequence of batch variation considerable doubt is thrown on the utility of assaying one batch of a particular brand as a means of determining the general potency of that particular manufacturer's product. Rickes assayed a number of American liver extracts of an alleged potency of either 10 or 15 U.S.P. units per ml. and found a marked variation between the various brands and also a difference between the various batches of the same brand. The American preparations which have been tested in these laboratories have ranged between 7.5 and

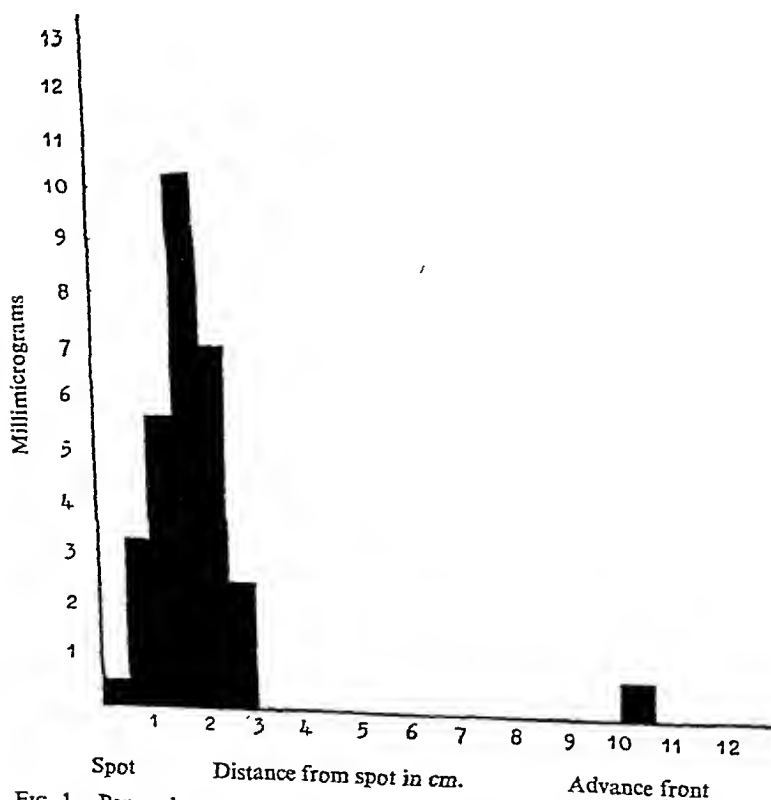


FIG. 1.—Paper chromatograph vitamin B₁₂ in liver extract product "B," 0.005 mL developed for 18 days.

13 $\mu\text{g.}$ of vitamin B_{12} per ml. with the exception of the 30 $\mu\text{g.}$ material already referred to. Manufacturer "F" issues 3 products, a liquid oral liver extract, a parenteral liver extract and a parenteral preparation labelled "Forte." All of these give exceptionally low values, and in this instance one is bound to suspect either the source of liver or else the primary extraction. In the case of manufacturer "E" and "B," both of which are highly reputable British firms, the explanation is not so obvious. Admittedly, one of the low-potency batches is known to be old, but others equally low were bought on the open market for current use. Moreover, we have been unable to find evidence that, in general, finished liver extracts deteriorate in potency on storage even when they have been stored under the adverse conditions which pertain in some of the export markets.

The only reasonable explanation is suggested by work in these laboratories concerned with investigating "process losses" by means of Dorner activity. It is now no secret that in a highly fractionated parenteral extract there may be very appreciable losses during processing, and that the magnitude of these losses may not be constant from sub-batch to sub-batch. Thus, for example, if a certain process is liable to give a 60 per cent. loss, it is not impossible that occasionally this loss is 90 per cent., so that unless a large number of sub-batches are combined in order to "iron out" the variations in process loss, the final product might be only one-third the potency of another.

SUMMARY

1. A number of commercial liver extracts have been assayed for *L. lactis* Dorner activity in terms of vitamin B_{12} . A considerable variation from brand to brand has been found and also a serious variation between different batches of the same brand.

2. Although steps have been taken to eliminate artefacts due to method and the presence of inhibitors, it is not possible at the present time to correlate exactly *L. lactis* Dorner activity with clinical efficacy.

3. It is thus premature to advocate the establishment of a "Dorner" assay unitage applicable to all liver extracts, but it is not too much to expect that, in the future, branded preparations will maintain a reasonably constant "Dorner" activity.

4. The presumptive vitamin B_{12} content of "high potency" liver extracts is of the order of 10 $\mu\text{g./ml.}$ whether of British, American or Continental manufacture.

It is a pleasure to acknowledge the technical assistance of Mr. G. B. D. Grafham.

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THE VITAMIN B₁₂ CONCENTRATION IN LIVER EXTRACTS AND A NOTE ON THE RELATIONSHIP BETWEEN CLINICAL RESPONSE AND B₁₂ DOSAGE

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VITAMIN B₁₂ CONTENT OF LIVER EXTRACTS

A NUMBER of different liver extracts have been assayed for vitamin B₁₂ activity by the cup-plate method of Cuthbertson¹ employing *Lactobacillus lactis* ATCC 8,000. Some of these samples have also been assayed by the technique of Lees and Emery² employing the tube assay with *Lactobacillus leichmannii* 313. The results obtained are summarised in Table I.

TABLE I
B₁₂ CONTENT OF LIVER EXTRACTS FOR PARENTERAL USE

B ₁₂ CONTENT OF LIVER EXTRACTS FOR PARENTERAL USE				
Distributor	Manufacturer	Extract	µg B ₁₂ /ml ‡	Potency
British	A	1	1.4	low
		2	5.0	high
	B	3	6.0	high
	C	4	2.9*	high
		5	0.8	low
	D	6	1.0*	medium
		7	12*	high
	E	8	3*	low
		9	12*	high
	F	10	0.2	high
European	G	11	(a) 6*	low
			(b) 3.4	low
			0.23	low
	H	12	2.5*	high
	I	13	0.5	high
	J	14	0.1	medium
15				
American	K	17	0.1	medium
			µg B ₁₂ < 0.05†	high
	L	18	2.7	low
			3.5	medium
	M	19	22	high
	N	20	1.8	high
	O	21	11.8	high
	P	22		

* Average of 2 to 8 different batches

† No B₁₂ detected even after chromatography

‡ The assay results given in TABLES I, III, IV and V represent the activity of one or more of the vitamin B₁₂ group of factors expressed as the concentration of standard vitamin B₁₂ (µg/ml) giving the same response as the sample

Samples used for assay are all recent, having been obtained for the most part in June, 1949, except for samples from manufacturer G of

which (a) were obtained during 1936 to 1939 and (b) was captured during the war. Of the above samples N, O and P are known to have been manufactured in America. The other samples whose manufacturers are described as European or American were nevertheless presumably made in England, since we are informed that no liver extracts have been imported into this country for the last 6 years. The description used is thus the country of the manufacturers' headquarters, but not necessarily of the extract's origin.

The extracts have been classified into groups of low, medium and high potency on the basis of information supplied with the extracts by their manufacturers. This classification, shown in Table II, is necessarily very rough in the absence of any standard method of describing potency, but in assigning the extracts to low, medium or high potency groups, the suggested dosing schedules, U.S.P. units and liver equivalents have been taken into consideration.

TABLE II

Potency	Stated liver equivalent ml.	U.S.P. units
Low	<10 g.	2
Medium	10 to 20 g.	10
High... ..	>20 g.	15

No attempt has been made to distinguish between highly refined and highly concentrated extracts, although it is obvious that some manufacturers have attempted to produce potent refined materials, while others appear merely to have concentrated their liver extracts without any great degree of purification.

Table I demonstrates the wide differences between extracts from different manufacturers, the low potency extracts ranging from 0.23 to 6 $\mu\text{g.}/\text{ml.}$, while medium and high potency extracts vary over the ranges of 0.1 to 3.6 $\mu\text{g.}$ $\text{B}_{12}/\text{ml.}$ respectively. These variations between samples of different origins would hardly be expected if adequate clinical trials had been carried out on all batches. This variation may be

TABLE III

BATCH TO BATCH VARIATION OF SAME BRAND OF EXTRACTS FROM DIFFERENT MANUFACTURERS

Manufacturer	Extract	Sample	$\mu\text{g. B}_{12}/\text{ml.}$	Manufacturer	Extract	Sample	$\mu\text{g. B}_{12}/\text{ml.}$
X	A	1	4.8	Z	C	12	15.0
		2	19.8			13	7.5
		3	11.3			14	10.0
Y	B	4	2.7			15	8.0
		5	3.6			16	5.0
		6	2.1			17	5.0
		7	3.0			18	15.0
		8	3.3			19	9.0
Z	C	9	24.0			20	17.5
		10	6.0			21	12.0
		11	9.0			22	12.0

VITAMIN B₁₂ CONCENTRATION IN LIVER EXTRACTS

ascribed to differences in manufacturing technique, quality of raw materials and clinical control (if any) of the products. Different batches of the same extract made by the same manufacturer may show a wide range of B₁₂ concentrations, but it cannot be said whether these may not be due to alterations in procedure and materials available for manufacture. Table III shows the degree of variation encountered.

From the results it is clear that little value can be attached to estimates of potency unless better control is used than apparently at present. A number of these samples have been assayed by both of the different techniques employed in these laboratories, with the results shown in Table IV.

TABLE IV

MICROBIOLOGICAL ASSAY OF PARENTERAL LIVER EXTRACTS BY CUP-PLATE (*L. LACTIS*) AND TUBE (*L. LEICHMANNII*) METHODS

Extract							Tube assay $\mu\text{g. B}_{12}/\text{ml.}$	Plate assay $\mu\text{g. B}_{12}/\text{ml.}$
1	0.05*	<0.1*
2	0.04*	<0.05*
3	0.6*	<0.1*
4	0.1*	<0.2*
5	0.14	0.22
6	0.4	0.8
7	0.7	1.0
8	1.5	1.4
9	2.8	2.7
10	3.0	3.5
11	3.3	3.3
12	9.7	10.5

* High concentrations of desoxyribosides present in these samples.

On the whole the agreement is reasonable for methods involving two different organisms and two different techniques (the cup-plate and tube method) having different sensitivities to interfering substances. The discrepancies encountered are being further investigated.

RELATIONSHIP BETWEEN CLINICAL RESPONSE AND MICROBIOLOGICAL ACTIVITY

All liver extracts prepared in these laboratories are clinically tested before they are released for sale. For this purpose typical cases of Addisonian pernicious anaemia in severe relapse are used, preferably those showing a red cell count between 1 and 2 million/cmm. The patients receive, by intramuscular injection, a single test dose of the extract. During the following 14 days the blood picture is determined on alternate days, but daily observations are made at the time when the peak of the reticulocyte response is expected. In interpreting the results particular attention is paid to the red cell response, which should increase at the rate expressed by the formula of $I = 0.94 - 0.214 E_0$ (Della Vida and Dyke³), where I is the weekly increment in the red cells and E_0 is the initial cell count. In assessing the response of an individual patient, consideration is given to other factors that may bear on the test, e.g., haemoglobin levels, shape of the response curves, reticu-

locyte response, infection and possible iron deficiency or abnormal red cell destruction. Foods that might have an anti-anæmic action are withheld before and during the period of test.

The results on 14 different liver extracts with 18 patients in a number of different hospitals are summarised in Table V, which gives only the red cell response as a percentage of that to be expected from the Della Vida and Dyke formula and the B_{12} content of the different extracts. The other observations made on these patients have been omitted for clarity.

If the responses of the individual patients are considered and if a response of 90 per cent. of that expected is taken as indicating activity of the liver extract, then it can be seen that samples containing less than

TABLE V
RELATION BETWEEN CLINICAL RESPONSE AND B_{12} ACTIVITY OF EXTRACT
(1 ML. OF EXTRACT USED FOR THESE TESTS)

Sample				B_{12} activity µg./ml.		Increase in red blood cells expressed as percentage of expected response		Sample				B_{12} activity µg./ml.		Increase in red blood cells expressed as percentage of expected response	
1	2.0		75		9	9.0		62	
2	2.9		nil 114	57	10	9.0		58	
3	3.0		nil		11	12.0		46 145	91
4	4.0		76		12	12.0		73 138	106
5	4.1		nil		13	15.0		93	
6	4.5		54		14	17.5		96 112	104
7	6.0		50									
8	6.0		109									

10 µg./ml. are much less satisfactory than those containing more than 10 µg./ml. Of the 11 patients receiving less than 10 µg. of B_{12} 9 gave unsatisfactory responses, while of the 7 patients who received more than 10 µg. only 2 gave unsatisfactory responses. Several of these extracts were tested on more than one patient. The marked variation in response from patient to patient is very clearly seen in the results obtained with these. In particular the different patients receiving samples 2 and 11 show very wide differences in response. If the results obtained with the two patients on each sample are averaged, then it can be seen that only one extract out of 10 containing less than 10 µg./ml. would satisfy our criterion (and this result depends on the reaction of only one patient) while none of the four extracts containing more than 10 µg. B_{12} /ml. would have failed to do so.

SUMMARY

1. The vitamin B₁₂ contents of a number of liver extracts have been reported.
2. Assay results using *Lactobaccillus lactis* and *L. leichmannii* have been compared.
3. The clinical responses to a number of extracts have been compared with their B₁₂ contents, and the range of activity found by clinical test has been indicated.

We wish to express our thanks to the pathologists without whose enthusiastic co-operation the standardisation of liver extracts would not have been possible.

REFERENCES

1. Cuthbertson. *Biochem. J.*, 1949. 44, v.
2. Lees and Emery. *Biochem. J.*, 1949. 45, ii.
3. Della Vida and Dyke. *Lancet*, 1942. 243, 275.

DISCUSSION

THE three papers dealing with the micro-biological assay of liver extracts by Mr. Shaw and by Dr. Cuthbertson, Miss Lloyd, Dr. Emery and Mr. Lees were discussed together; the last paper was read by Mr. Lees.

MR. SHAW, in presenting his papers, stated that since submitting them he had found that on applying his assay to a commercial solution of crystalline vitamin B₁₂ prepared for injection, the indicated vitamin B₁₂ content appeared to be approximately three times as great as the labelled value, using as reference standard the solid liver preparation supplied by Dr. Rickes and standardised by him at 0.4 µg. per mg. This observation indicated that the results quoted in the paper represented not necessarily vitamin B₁₂ as such but were a measure of the growth activity for *Lactobaccillus lactis* Dorner. This discrepancy along with the extremely slow speed of migration of the main constituent on paper chromatography, and the American view that microbiological assay of liver extracts for vitamin B₁₂ is not reliable unless the vitamin B₁₂ content of the liver solids in the preparation under test is of the order of 50 per cent. suggests the possibility that the clinical action of liver extracts may be due to a complex or conjugate of vitamin B₁₂ more than to the presence of the free vitamin. It might well be that for the assay of liver extracts a standard liver preparation will be a more satisfactory reference standard than pure crystalline vitamin B₁₂.

The CHAIRMAN said that the three papers dealt with a subject which had been developed very considerably in the last year or so. If the figures given for assays of commercial extracts really represented their content of vitamin B₁₂ then he thought that they gave a very disturbing picture of the state of affairs. The assay process required improvement before it was possible to place reliance on it, but the results given in the papers suggested that there might be some relationship between it and the clinical re-

Mr. Shaw used a method of paper strip chromato-

graphy which was unusual, in that he did not protect the solvent from evaporation.

DR. C. H. HAMPSHIRE (London) commenting on the paper by Dr. Cuthbertson and his collaborators, remarked that he was a little sorry to see that they still found it necessary to make reference to the U.S.P. unit. The sooner that term disappeared the better. It was to be hoped that the work which was now being done by Dr. Cuthbertson and his colleagues and by other teams would enable a truly scientific method of assay of liver extracts to be arrived at by comparison with a standard.

DR. F. HARTLEY (London) said that the papers would be generally welcomed as helping to show something of the progress made on the microbiological side since the isolation of vitamin B₁₂ last year, but a comparison of the two sets of papers as circulated revealed some startling discrepancies. However, Mr. Shaw had now clarified some of those discrepancies. The variations which became apparent immediately the papers were read would obviously be regarded by many people as being attributable to the essentially variant factors present in the microbiological method, namely the standard, the test organism, and the medium used. Mr. Shaw in his abstract, but not in the paper, indicated the very different interpretation to be given to the results, when assaying preparations of pure vitamin B₁₂ in contrast to those for the other materials examined. The standard which he used contained 0.4 µg./mg., or in other words 0.04 per cent. of vitamin B₁₂. It was well known, as Mr. Shaw himself had pointed out, that liver preparations contain other growth factors than vitamin B₁₂, and a relationship between the growth factors in the standard and in the test could be obtained. Mr. Shaw provided histograms for materials under test but not for the material used as his standard; however, he had pointed out that when he measured pure vitamin B₁₂ against his standard he got a result about three times larger than he had expected. Therefore, making that allowance, the results of the two sets of results came more nearly into line. The marked influence of the medium on the growth-promoting and growth-inhibiting activities, and therefore on the results obtained, was fully discussed by Dr. Mary Shorb at the recent International Biochemical Congress. It became clear on looking at her growth response slopes that, according to factors such as would occur when comparing materials of totally different origin, some of which contained conjugates and others free vitamin B₁₂ that one would obtain different interpretations according to which slope was used. To compare one kind of material with others of a different nature might be grossly misleading. It was now well known that different strains of organism responded differently to a particular standard and a particular unknown. Dr. Cuthbertson and his colleagues had suggested in Table IV that comparable results were obtained using *Lactobacillus lactis* ATCC 8,000 and *Lactobacillus leichmannii* 7,830 for tube assay, but other strains were available, and if the authors would look at that most recently made available, *L. lactis* Dorner ATCC 10,697, they would find a rather different picture. Both Jukes and Lester Smith had shown that there might be a group of vitamin B₁₂ factors and Jukes had

already obtained in crystalline form a second vitamin B₁₂ which he had called vitamin B_{12b}. Some or all of these different factors might well be clinically active. Mr. Lees had been understandably cautious in his interpretation of Table V, but it would be seen that 1 ml. of the extract containing 2.9 µg./ml. of vitamin B₁₂ gave in one patient a response of a similar order to that given in another patient receiving 1 ml. of an extract containing 17.5 µg./ml. Such a contrast strongly suggested that there might be some factor additional to vitamin B₁₂ exerting an erythropoietic effect. Standardisation in terms of vitamin B₁₂ only, might not, therefore, wholly reflect the clinical potency of liver preparations. Mr. Shaw had suggested in his abstract, but not in his paper, that it might be desirable to assay the liver extract not against vitamin B₁₂ but against a standard liver preparation. That was certainly a wise extension; but the difficulty lay in arriving at a standard preparation. Were they interested in the conjugated vitamin B₁₂ or in free vitamin B₁₂? He thought it was important to obtain a great deal more information on the factors which could be separated from liver extracts chromatographically, and not necessarily limited to the coloured fractions. Under the conditions for chromatography which Mr. Shaw had described, most workers would have lost all their vitamin B₁₂. It would normally have moved far more than 2.5 cm. up the paper during elution for 18 days; overnight elution was sufficient for most people. Finally, it was important to recognise that if in fact the authors had measured growth-promoting factors for an organism and not vitamin B₁₂ there should be revision of the headings in the tables in the papers.

DR. K. BULLOCK (Manchester) asked about the effects of autoclaving on the glucose. Mr. Shaw had said that the lactobacillus was very sensitive to the concentration of carbon dioxide and then later that autoclaving altered the redox potential. Were those two factors standardised in the medium which he recommended and stabilised during the method of preparation?

MR. T. D. WHITTET (London) commented that almost every physician had his favourite extract and pharmacists therefore had to stock a great variety of liver extracts. He inquired whether it had now been proved that both crude and refined liver extracts were necessary.

DR. G. E. FOSTER (Dartford) asked whether the culture could be kept in the dried state.

DR. LUMB (Nottingham) enquired whether there had been any attempt to remove the thymidine before carrying out the assays. If not, to what extent was the thymidine responsible for the growth of the lactobacillus?

MR. G. E. SHAW, in reply, said that the histogram of his standard was identical with the histogram of the extracts, there was no marked separation of the slow moving band. He had tried a normal downward development and had obtained exactly the same kind of histogram. It was owing to that slow moving band that he was beginning to wonder whether in an ordinary liver extract there was much free crystalline

vitamin B₁₂. In the course of work not yet published he had examined liver extracts from animals treated in certain ways. Two extracts, one from a treated and one from a normal animal, were adjusted to the same nominal vitamin B₁₂ concentration. The normal one showed a normal crude liver extract histogram, but the other had multiple peaks. The normal one gave the expected clinical response, but the second one gave no response whatever. The mathematical formula used in the paper read by Mr. Lees was not an assay but a stipulation that a case which had been satisfactorily treated would on average give that response. There was no evidence whatever that the same dose of liver extract would give the same response in two patients, even if they had the same initial r.b.c. level. Apart from indicating that a weak liver extract was less likely to give the response than a strong one, he did not think that any reliance could be placed on the formula.

L. leichmannii, which was more subject to oxygen tension than *L. lactis*, Dorner was difficult to maintain under aerobic conditions. It must be grown on a medium in which the glucose had been autoclaved. He exposed the medium to air for 3 or more days after manufacture in order to enable it to reach equilibrium. He had also used a series of tubes which had been autoclaved six months before, or earlier, and had obtained a growth response curve identical with that which he had obtained six months before.

MR. K. A. LEES, also in reply, said that as the U.S.P. authorities had stated that they were not prepared to accept standardisation in terms of vitamin B₁₂, but only to accept the U.S.P. unit, it was necessary at this stage to incorporate the U.S.P. unit for purposes of comparison in any paper. He would like to thank Dr. Hartley for his suggestion with regard to *L. lactis* Dorner, ATCC 10, 697. With reference to redox potential, contrary to what Mr. Shaw stated, they had found that with *L. leichmannii* they could obtain satisfactory growth whether or not the medium was autoclaved containing glucose. They might get better growth when the glucose was autoclaved with the medium, but even if it was added as a sterile solution they still obtained the same result. The solution to the problem would be to determine the optimum E_H for initiation of growth, and to find a means of stabilising the medium at that particular E_H . Various methods had been suggested in the literature and they were examining them at the moment, particularly the effect of adding thioglycollic or ascorbic acid to the medium. Autoclaving thioglycollic acid and vitamin B₁₂ together in the basal medium might result in a similar growth response to that obtained with aseptic addition of vitamin B₁₂ to a sterile medium not containing thioglycollic acid, due to the growth response of thioglycollic acid compensating for any destruction of vitamin B₁₂. He did not think that it was possible to say at the present time whether there was any advantage in using a crude extract, except that Table I showed that in the more refined extracts the vitamin B₁₂ activity tended to be higher than in the other extracts, though it could also be just as low.

MICROBIOLOGICAL ASSAYS; VITAMIN B₁₂

Both Dorner and the *L. leichmannii* cultures could be dried, but when resuscitating the culture it was 7 to 14 days before a satisfactory growth response could be obtained.

They had not attempted to remove thymidine. They did a differential assay, determining vitamin B₁₂ plus thymidine and then thymidine alone, after alkaline hydrolysis of the vitamin B₁₂. The Della Vida and Dyke formula was compiled from a large number of clinical tests, and so far had been the only scientific approach to clinical testing. As such, they had considered it to be the most suitable standard for their present work. He, personally, agreed with Dr. Hartley that 18 days was a long time for developing the chromatogram: they found that 24 hours was quite adequate. It was difficult to say whether the vitamin B₁₂ content reflected the clinical activity. Medical opinion accepted vitamin B₁₂ activity as the total anti-anæmia activity, but the scientific mind did not.

THE VARIATIONS IN COLOUR OF LIQUID EXTRACT OF LIQUORICE B.P.

By J. H. OAKLEY AND R. E. STUCKEY

From The British Drug Houses Ltd., London, N.1

Received July 1, 1949

It is well known that variations in colour are found between batches of liquid extract of liquorice B.P. prepared from different samples of liquorice root.

Colour differences in liquid liquorice extracts are usually noticed in the dispensing of mixtures having the extracts as a constituent; the dilution obtained in such mixtures is often such as to bring it within what might be called the "critical" colour range. Variations in colour are not readily apparent until a dilution of 1 in 10 is reached, but differences can be detected in dispensing a number of common National Formulary mixtures. Thus *Mistura Ammonii Chloridi* contains a 15 minim dose and *Mistura Ammoniae et Ipecacuanhae Composita* a 10 minim dose of liquid extract of liquorice; these amounts represent dilutions of 1 in 16, and 1 in 24 respectively, and variations in the colour of the liquid extract of liquorice used will result in a different colour for the final dispensed mixtures, which may cause comment. In an endeavour to trace the reasons for these variations, details of the method of preparation have been examined, together with differences inherent in the drug and in the final product.

The British Pharmacopœia 1948 directs that unpeeled liquorice root, *Glycyrrhiza glabra* and other species of *Glycyrrhiza* shall be used in the preparation of liquid extract of liquorice. There are considerable differences in commercial samples of liquorice root and Wallis¹ gives figures of 15 to 27 per cent. for aqueous extractive, dried at 100°C.; the B.P. excludes samples of root with a water-soluble extractive below 20 per cent. Liquorice root of commerce is at present imported mainly from Anatolia, Syria or Iraq as ordinary or "natural" root; another type available is known as "cuttings" which are selected pieces of uniform

TABLE I
ANALYSIS OF COMMERCIAL SAMPLES OF LIQUORICE ROOT

Description of material	Moisture	Ash	Acid-insoluble ash	Water-soluble extractive (on material as received)
	per cent	per cent	per cent	per cent
Natural, unpeeled	8.3	4.4	0.4	20.5
Cuttings, unpeeled	8.6	5.1	0.6	31.6
Powdered decorticated*	7.0	3.9	0.1	41.2
Cuttings, unpeeled	9.1	6.9	0.6	35.3
Natural, unpeeled	10.7	5.4	0.6	25.1
Cuttings, unpeeled	8.5	5.2	0.2	33.1
Natural, unpeeled	8.4	7.6	0.9	25.9
Natural, unpeeled	7.0	5.1	0.3	21.0

* Described as "pulv. decort. elect."

VARIATIONS IN COLOUR OF LIQUID EXTRACT OF LIQUORICE B.P.

size and diameter, the length of each piece being approximately 2 to 4 inches. A selection of samples of liquorice root obtained from different sources during recent years gave the analytical results shown in Table I and indicate the variations to be expected.

The figures are chiefly of interest in showing the wide variations in water-soluble extractive encountered in different samples of the crude drug; the remarkably high extractive in the single sample of commercial powdered decorticated root is also noteworthy. The method used for the determination of water-soluble extractive was similar to that described in the B.P. 1948.

Examination of the colouring matter

Representative samples of each of 6 lots of liquorice root, which complied with the requirements of the B.P. 1948, were reduced to a coarse powder and macerated with chloroform water (5 g. root to 100 ml.) for 24 hours, shaking frequently. The resulting product was filtered and aliquot portions of the filtrate were taken for determination of the water-soluble extractive and also for recording the colour in Lovibond units in a Lovibond Tintometer using a 1 cm. cell. The results obtained are given in Table II.

TABLE II
COLOUR VALUES OF SAMPLES OF LIQUORICE ROOT

Sample	Source and description of root	Water-soluble extractive	Lovibond Units using a 1 cm. cell			
			Yellow	Red	- Blue	Neutral
		per cent				
A	Anatolian, natural	35.9	9.6	5.1	—	—
B	Iraq, natural	26.9	10.1	6.2	—	0.1
C	Syrian, natural	20.5	11.0	5.7	—	0.2
D	Anatolian, cuttings	32.3	11.5	4.5	—	0.2
E	Anatolian, cuttings	33.7	11.0	4.2	—	—
F	Anatolian, cuttings	34.5	11.6	5.2	—	0.2

It can be seen from Table II that although the water-soluble extractive varied from 20.5 per cent. in sample C to 35.9 per cent. in sample A, the colour of the extracts did not vary appreciably; the composition and intensity of colour expressed as above were in fact relatively constant, despite the differences in origin and characteristics of the samples. Thus, under the conditions of experiment used, it can be concluded that the "depth" (composition and intensity) of colour extracted per unit weight of root appears to bear no relation to the percentage of water-soluble extractive obtainable from the material.

Usually most samples of extract of liquorice throw out a precipitate when added to acid solutions. For the preparation of liquorice extracts a dilute ammonia solution may be used in the process as, for example, in the preparation of liquorice root fluid extract, U.S.P. XIII, and Berg² used a menstruum containing ammonia for the extraction of liquorice by percolation. To study the effects on the resultant colour, further 5 g. quantities of the materials reported in Table II were macerated using a dilute (1 per cent. w/w) solution of ammonia in place of chloro-

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Cuttings, unpeeled	9.1	6.9	0.6	35.3
Natural, unpeeled	10.7	5.4	0.6	25.1
Cuttings, unpeeled	8.5	5.2	0.2	33.1
Natural, unpeeled	8.4	7.6	0.9	25.9
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			Yellow	Red	- Blue	Neutral
		per cent.				
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B.	Iraq, natural	26.9	10.1	6.2	—	0.1
C.	Syrian, natural	20.5	11.0	5.7	—	0.2
D.	Anatolian, cuttings	32.3	11.5	4.5	—	0.2
E.	Anatolian, cuttings	33.7	11.0	4.2	—	—
F.	Anatolian, cuttings	34.5	11.6	5.2	—	0.2

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form water. The proportion of extractive obtained was determined and the colour examined as before. The results obtained are given in Table III.

TABLE III
EXTRACTIVES AND COLOUR OF EXTRACTS OBTAINED WITH A DILUTE SOLUTION OF AMMONIA

Sample of Liquorice Root (see Table II)	Dilute Ammonia-soluble extractive	Lowbond Units using a 0.25 cm cell			
		Yellow	Red	Blue	Neutral
	per cent				
A	34.5	12.3	3.8	—	0.1
B	29.5	10.0	3.5	—	—
C	21.8	13.4	5.5	—	—
D	33.5	13.2	5.0	—	—
E	36.4	19.0	3.3	—	0.2
F	34.5	18.2	4.4	—	0.2

The use of dilute solution of ammonia did not increase significantly the total percentage of extractive, as can be seen by comparison of the extractive figures for the respective samples given in Table II and Table III.

It should be noted, however, having regard to the smaller cell width used, that the intensity of colour of the extract as expressed had been very appreciably increased. The question arises, therefore, whether the increased intensity of colour results from the extraction of additional coloured materials by the alkaline solution or is simply due to a darkening or "indicator" effect at alkaline pH values.

A sample of aqueous extract (5 g. of liquorice root, 100 ml. of water) was therefore adjusted to various pH values by the addition of small amounts of either sodium hydroxide solution or hydrochloric acid and the colour examined (see Table IV). The pH value of the extracts before addition of acid or alkali was approximately 6.5.

TABLE IV
EFFECT OF pH ON THE COLOUR OF AN AQUEOUS EXTRACT OF LIQUORICE ROOT

pH Value of extract after addition of acid or alkali	Lowbond Units using a 0.25 cm cell			
	Yellow	Red	Blue	Neutral
2.0		Cloudy solutions		
4.0				
6.5	2.3	0.7	—	—
7.0	4.0	1.0	—	—
7.5	4.9	1.0	—	0.1
8.5	6.0	1.6	—	0.1
9.0	9.0	1.9	—	0.2
9.5	13.9	2.4	—	0.3
10.0	16.0	3.0	—	0.1

In acid solutions (low pH values) precipitation occurred which interfered with the examination of the colour of the liquid. It can be seen, however, that as the pH increased and especially on the alkaline side the extracts became considerably darker, both yellow and red components increasing in intensity. The colour obtained at each pH value was stable.

VARIATIONS IN COLOUR OF LIQUID EXTRACT OF LIQUORICE B.P.

however, and showed no significant change over a period of two days. It was noted incidentally that on the addition of alkali a buffering effect occurred between pH 8.5 and 9.0 and the addition of a relatively large quantity of sodium hydroxide solution was required to increase the pH above 8.5.

Because of the difficulty of measuring directly the colour of the extracts at low pH values, the effect of acid and alkali on the colour isolated chromatographically was examined. A sample of aqueous extract of liquorice root was passed through a column of alumina when the colour was adsorbed. Washing down the column with quantities of water then produced a dilute aqueous solution of the coloured materials of the extract substantially free from other water-soluble matter. Aliquot quantities of this solution were then adjusted to various pH values and the colour examined. The results obtained are given in Table V.

It can be seen that there is a definite "indicator effect" obtainable with the colouring matter from extract of liquorice, the colour being much lighter in acid and darker in alkaline solution. This effect was found to be reversible. Addition of alkali to an acidified solution quantitatively restored the colour, and addition of acid to an alkaline solution diminished the colour, which could be restored on making alkaline once more.

TABLE V
EFFECT OF pH ON THE COLOUR OF AN AQUEOUS SOLUTION OF THE
COLOURING MATTER ISOLATED FROM AN EXTRACT OF LIQUORICE ROOT

pH	Lovibond Units using a 0.25 cm. cell			
	Yellow	Red	Blue	Neutral
2.0	0.4	—	—	—
4.0	0.8	—	—	—
6.5	1.0	—	—	—
8.0	1.1	—	—	0.2
9.0	2.4	0.1	—	0.1
10.0	2.7	—	—	—
10.5	2.8	—	—	—

It will be realised from the results reported that, according to the conditions of preparation and use of liquid extract of liquorice B.P. so there can result variations in the colour of the products.

After evaporation according to the official instructions a pH slightly on the acid side is usually obtained, but the variation of acidity encountered is not such as to result in appreciable variations in colour due to the indicator effect referred to above. Since, however, the B.P. instructs "evaporate until the weight per ml. of the liquid at 20°C. is 1.198" it follows that the standardisation of the liquid extract on total water-soluble extractive, though entirely logical for such a product, may cause relatively appreciable differences in colour between extracts prepared from samples of root showing a relatively high, and from those showing a relatively low, water-soluble extractive. Thus, for example, a sample of liquorice root giving a high water-soluble extractive will give a comparatively large volume of final product and since, as was shown in

Table II, the amount of coloured materials extracted is independent of the water-soluble extractive of the root, the resulting preparation will be relatively light in colour. Using water-soluble extractive as a criterion of quality of liquorice root it follows that a good quality root may, in fact, result in an official extract relatively light in colour. The yields and final colours of a number of prepared samples of liquid extract of liquorice B.P. were therefore noted and these are given in Table VI; the recorded yields were obtained using B.P. quantities and the Lovibond Tintometer readings are for 1 in 100 aqueous dilutions of the liquid extract. These results show that a high yield was associated in these examples with a relatively low colour intensity. Having regard, however, to the variations encountered between laboratory and industrial methods of evaporation and to lack of knowledge of the influence of heat on the constituents of the extract responsible for its colour, it would not be justifiable to expect such a relationship to be applicable generally.

TABLE VI
CORRELATION BETWEEN YIELD AND COLOUR FOR SAMPLES OF LIQUID
EXTRACT OF LIQUORICE B.P.

Example	Yield	Lovibond Units using a 1 cm. cell			
		Yellow	Red	Blue	Neutral
	ml. per 1000 g. of root				
1	...	481	14.1	1.7	—
2	...	678	7.0	0.9	—
3	...	566	10.3	1.3	—
4	...	655	7.0	0.9	—
5	...	368	14.2	1.8	—
6	...	671	7.5	0.9	—
7	...	716	5.4	0.7	—
8	...	569	6.5	0.8	—

It is interesting to note that, while the British Pharmacopœia 1948 adopts the more logical procedure of standardisation on solid content, thus leading to the possibility of variations in colour according to quality of root used, the method of the United States Pharmacopœia XIII will lead to the production of extracts varying in solid content according to the water-soluble extractive of root used, but the extracts obtained are likely to be relatively constant in colour.

The colour measurements given in Table VI are, as mentioned, those obtained in aqueous dilutions only. The colour in dispensed mixtures will, of course, differ according to a number of factors as well as the pH of the solution; for example, the nature and concentration of the electrolytes present may also cause alterations in colour.

Certain Aspects arising during Manufacture. In the preparation of the liquid extract the B.P. directs that unpeeled liquorice root, in coarse powder, shall be exhausted by percolation with chloroform water, the percolate boiled and set aside for not less than 12 hours, the clear liquid decanted and the remainder filtered from the relatively light-coloured sludge present in the latter being rejected. This sludge is not sufficiently coloured, however, to affect the colour of the final preparation. It is not

VARIATIONS IN COLOUR OF LIQUID EXTRACT OF LIQUORICE B.P.

advisable to set aside the preparation for any length of time at this stage, owing to the likelihood of fermentation. In practice on the manufacturing scale, evaporation may be done under reduced pressure so that changes in the colour of the product due to overheating are less likely to occur.

As it was thought possible that differences in the time for which the preparation is allowed to stand before filtration (the B.P. states "not less than forty-eight hours") might cause slight differences in the colour of the final preparation, the effect of time of standing was examined. After standing for three weeks a small heavier layer collected, giving the following comparative results (1 in 100 dilution, Lovibond Units):

Extract immediately after preparation:	Yellow 8.0; Red 1.0
Extract (lower layer) after standing for three weeks	Yellow 10.6; Red 1.3

Although, as can be seen, the lower layer was relatively more intensely coloured, the actual volume of this layer was small in comparison with the bulk of the preparation and the amount, later rejected, did not appreciably affect the colour of the bulk of the extract.

SUMMARY

1. A study has been made of the colouring matter extracted from liquorice root and of some of the possible causes of variation in colour of liquid extract of liquorice B.P.

2. It has been shown that the colour variation occurring in samples of extract prepared according to the official instructions may result from the standardisation of the preparation on its content of total water-soluble extractive.

The effect of changes in pH value on the colour of liquid extract of liquorice has also been investigated.

The authors wish to thank Miss S. M. Stokes for assistance in carrying out the practical work, and the Directors of The British Drug Houses, Ltd., for permission to publish this paper.

REFERENCES

1. Wallis, *Textbook of Pharmacognosy*, 1946, Churchill, 334.
2. Berg, *J. Amer. pharm. Ass., Sci. Ed.*, 1924, 13, 814.

DISCUSSION

The paper was read by MR. J. H. OAKLEY.

THE CHAIRMAN said that it was interesting to note the increasing use of chromatography, which was coming into a large number of Conference papers.

DR. J. M. ROWSON (London) remarked that nothing seemed to be known about the nature of the colouring matter present in liquorice root and the standard textbooks made virtually no reference to it. He wondered whether the authors had any idea of the chemical nature of

the colouring matter present. Did they consider that there was any possibility of caramelisation of the fairly large amount of sugar present? That in itself would tend to account for the darker colour of the extracts which had been prepared.

DR. W. MITCHELL (London) said that, although he had not made any colour measurements himself he assumed, by analogy with other cases that the red component was the dominant one in controlling the colour, and that the yellow was of relatively small importance. He wondered, therefore, whether the figures given in Table V were of any value, because, in exposing the material to chromatographic separation all the red component had been lost and one was merely measuring the yellow. Also, there was a buffer effect on the pH values corresponding to the very large change in the colour values at just about the same point. His experience did not confirm the wide variations in colour found by the authors. It was also necessary to consider the effect of contact with metals in large manufacture. Experiments with glass apparatus were not entirely realistic. A good deal might depend on the metal used in the plant, and even more on the heat treatment which the material had undergone. Caramelisation might play a very significant part in determining the final colour of the product, and it was most important to avoid the risk of overheating at any stage.

MR. J. H. OAKLEY, in reply, said that the authors had no information about the nature of the colouring matter present. In manufacturing practice caramelisation was not likely to be considerable, as it was customary to do the evaporation under reduced pressure. They had deliberately tried to produce a certain amount of caramelisation, and a small sample of extract was grossly overheated until actual charring occurred. They were surprised to find that very little increase in colour resulted.

They considered that the yellow was the more dominant factor and not the red. The buffer effect referred to was interesting. It was possible that some change took place at that stage, as it was coincident with a marked change in colour occurring there. Dr. Mitchell's somewhat different experience regarding the magnitude of the variation in colour might be because, to get a relatively constant colour, it was necessary to use the same type of root. If, however, roots of low and high water-soluble extractive were used a considerable variation in colour occurred. Though much of the work described had been done on a small scale in glass apparatus, it had also been compared with large-scale experiments done in large apparatus of various metals, and they had found no significant change in the colour.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Aldehydes, Determination of, with Hydrazine. L. Fuchs and O. Matzke. (*Scientia pharm.*, 1949, 17, 1.) Hydrazine sulphate may be used for the acidimetric determination of aldehydes, details being similar to that of the hydroxylamine method, with methyl red as indicator. Examples are given of the application to benzaldehyde, piperonal, and vanillin.

G. M.

Atropine, Determination of Small Concentrations of. M. Tonnescn. (*Acta Pharmacol. Toxicol.*, 1948, 4, 186.) The colorimetric reaction of Vitali as modified by Allport and Wilson is recommended for the chemical determination of small concentrations (50 μg . or more) of atropine, although the substance must be fairly pure. The conditions and precautions necessary to make the reaction quantitative are discussed and standard curves of extinction and time are given. Details of a biological assay depending on the dilatation of the mouse's eye are described, the technique being based on the method worked out by Pulewka (*Arch exp. Path. Pharmacol.*, 1935, 178, 439). The alkaloid is injected subcutaneously into each of 10 male white mice in an amount that in mydriatic effect corresponds to 0.75 μg . of *l*-hyoscyamine and the pupil diameter is measured microscopically after 1 hour. Dose response curves are given for atropine sulphate, hyoscyamine sulphate and scopolamine hydrobromide. A statistical analysis of results obtained using less than 10 mice has been made. After a large number of determinations it was found that a pupil diameter of 2 mm. corresponded to a minimum of 1.75 μg . and a maximum of 2.0 μg . of atropine sulphate. In forensic analysis an ether extract from an alkaline solution of the urine should be made evaporated to dryness, the residue dissolved in 0.001N hydrochloric acid and injected. An extraction of the contents of stomach and intestines with 0.001N hydrochloric acid should also be made, this procedure being more reliable than the "Stas-Otto" process. The untreated urine can also be injected. Amounts as small as 0.5 μg . can be determined by this method.

R. E.S.

Digitoxin and Digitoxigenin; Baljet Reaction. F. K. Bell and J. C. Krantz, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, 38, 107.) The Baljet test was applied using the original method, in which sodium hydroxide is the alkali, and the modified method, in which tetraethylammonium hydroxide is the alkali. The results indicate that under the conditions described there is no difference on a molar basis in the sensitivities of digitoxin and digitoxigenin towards the reagent, particularly if allowance is made for the digitoxose portion of digitoxin. If, therefore, digitoxin contaminated with digitoxigenin, which is physiologically less active, is assayed by the method described, erroneously high results will be obtained. Comparison of the results of the two methods supported the previous conclusions that the use of tetraethylammonium hydroxide as the alkali resulted in an increase of about 100 per cent. in the colour intensity.

G. R. K.

ABSTRACTS

Histamine, Microchemical Identification of Small Quantities of. G. Deniges. (*Bull. Soc. Pharm. Bordeaux*, 1949, 1, 87, 3.) A solution of histamine dihydrochloride was used to study the microcrystalline reactions of histamine. A small drop of a solution (concentration, 0.1 mg./ml.) containing 0.0003 mg. of histamine was allowed to crystallise spontaneously in air, when a number of octahedral crystals and aggregates were obtained. These were examined microscopically and a diagram at a magnification of 130 diameters is given. To the crystals was added a small drop of a 1 per cent. aqueous solution of picric acid; at the edge of this drop bundles of fine yellow needles of histamine picrate formed, not to be confused with the picric acid crystals, larger and more squat, due to excess of picric acid which crystallised out on standing. The presence of chloride in the evaporated crystalline histamine dihydrochloride can be confirmed by the addition of a small drop of silver nitrate solution; the precipitated silver chloride was dissolved in ammonia solution and allowed to evaporate, when characteristic crystals of silver chloride were formed.

R. E. S.

Pamaquin, Colorimetric Detection and Determination of. J. A. Sanchez. (*Ann. pharm. franc.*, 1949, 6, 495.) Three reactions are described. *Quinone reaction.* On warming a few mg. with 2 drops of 33 per cent. sulphuric acid in a glycerin bath at 155° to 160°C. for 5 minutes, a carmine-red colour appears on the walls and bottom of the tube. This is soluble in alcohol (70 per cent.) and becomes violet with ammonia. Tablets should be extracted with alcohol before the reaction is applied. *Nitrous acid reaction:* A few mg. is dissolved in 2 ml. of alcohol (70 per cent.) and treated with 2 drops of acetic acid and 1 drop of 10 per cent. sodium nitrite solution. A red colour is produced. On diluting and extracting with chloroform the colour is extracted by the chloroform. *Diazo reaction.* A red colour is produced by coupling with diazotised *p*-nitraniline. This reaction may be used quantitatively as follows: Dissolve 0.01 g. with 10 drops of acetic acid in alcohol (95 per cent.) to 100 ml. To 1 ml. of this solution 0.5 ml. of diazotised *p*-nitraniline solution (0.5 g. of *p*-nitraniline and 0.5 ml. of sulphuric acid in 50 ml. of water) is added; to 7 ml. of this solution 1 drop of 10 per cent. sodium nitrite solution is added, and the mixture is shaken until decolorised. The volume is finally made up to 5 ml. with alcohol (95 per cent.). For tablets, the reaction should be applied to the alcoholic extract.

G. M.

ESSENTIAL OILS

Ascaridol in Oil of Chenopodium. A. Halpern. (*J. Amer. pharm. Ass., Sci., Ed.*, 1948, 37, 161.) Ascaridol is the only anthelmintic component of oil of chenopodium, but the U.S.P. X, gave no standard for ascaridol content, other than physical constants. A method of determination based on the oxidation of ascorbic acid in the presence of ascaridol and of oil of chenopodium, and subsequent back titration of the ascorbic acid with 2:6-dichlorophenolindophenol has been investigated. Uniform results were not obtained, indicating that some other substance in the oil also caused oxidation.

L. H. P.

FIXED OILS, FATS AND WAXES

Shark Liver Oil, Deodorisation of. P. K. Mathew, P. V. Nair, T. A. Ramakrishnan and H. Sreemulanathan. (*Nature*, 1948, 162, 494.) Methods of deodorisation of shark liver oil are examined. Steam treatment of the oil yields a product free from odour when freshly prepared but which reverts to its original character in a few days. Oils

deodorised by agitation with fermenting milk or toddy, were found to remain bland for several months. Tables giving data with regard to the chemical constants, potency, and stability of oils deodorised with fermenting milk and toddy, as compared with those of the original oil, showed that these characteristics were little changed in the process. Hydrogenation of the oil was carried out with 0.1, 0.25 and 0.4 per cent. of a nickel catalyst with promising results; 0.1 per cent. of catalyst was not sufficient to effect complete deodorisation within a temperature range of 100° to 180° C., but 0.25 per cent. of the catalyst effected fairly complete deodorisation of the oil at 120° C. within 30 to 45 minutes. A loss of vitamin A of about 7 per cent. was found although the keeping quality of the oil improved considerably. Increase of the concentration of the catalyst to 0.4 per cent. caused increased destruction of vitamin A. Hydrogenation results are given in a table which shows that with 0.25 per cent. of nickel catalyst at temperatures between 100° and 180°C. and reaction periods from 30 to 180 minutes, the loss of vitamin A increased progressively with rise in temperature and time of exposure.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Biocerin. C. W. Johnson, H. D. West, H. L. Jones and C. J. Long. (*J. Bact.*, 1949, 57, 63.) The antibiotic material was obtained from a culture of *Bacillus cereus* isolated from soil and grown in a medium containing glucose, mineral salts, methionine and agar, by extracting the metabolism solution with ether and evaporating the ethereal liquid. The residue was insoluble in water but soluble in various organic solvents. The growth of all the following bacteria was inhibited by a concentration of 1 mg./ml. of the crude material, and all except the first three by 0.5 mg./ml.—*Salmonella paratyphi A and B*, *Sarcina lutea*, *Salmonella typhosa*, *Corynebacterium diphtheriae*, *Bacillus anthracis*, *B. subtilis*, *Staphylococcus albus*, *Escherichia coli*, *Brucella suis*, *Aerobacter aerogenes* and *Neisseria catarrhalis*. Rabbit serum caused a lowering of activity. A dose of 20 mg. in liquid paraffin suspension given intraperitoneally to mice did not produce any toxic effects. Further investigation is considered to be warranted.

H. T. B.

Circulin, A New Antibiotic. F. J. Murray, P. A. Tetrault, O. W. Kaufmann, H. Koffler, D. H. Peterson and D. R. Colingsworth. (*J. Bact.*, 1949, 57, 305.) The antibiotic was isolated from an aerated broth culture of a soil organism resembling *Bacillus circulans*, but differing from almost all known strains of the latter by giving a positive Voges-Proskauer reaction. The unit of activity is defined as that amount per ml that completely inhibits the growth of *Salmonella typhosa* for 18 hours at 37° C. in a broth containing 0.75 per cent. of peptone and 0.25 per cent. of yeast extract at pH 7.2. Material of the highest potency contained about 5000 units/mg. Circulin sulphate is exceedingly soluble in water, less soluble in the lower alcohols and insoluble in hydrocarbons or ether. It is not affected by autoclaving for 15 minutes. It is more active against Gram-negative than against Gram-positive organisms, thus differing from all other-

antibiotics except aerosporin and polymyxin. In general, circulin is more active than streptomycin against Gram-negative bacteria. Doses of 27 units/kg. subcutaneously, protected mice against 1000 minimum lethal dose of *S. typhosa*; 43,200 units per kg. intraperitoneally gave protection against 100 minimum lethal doses of *Klebsiella pneumoniae*. It is less toxic than aerosporin but more so than polymyxin. The LD₅₀ in mice for a preparation about 50 per cent. pure given subcutaneously is about 150 mg./kg.

H. T. F.

Penicillin, Antibacterial Activity of Synthetic Compounds of. Brownlee and M. Woodbine. (*Brit. J. Pharmacol.*, 1948, 3, 30) The compounds examined consisted of the following groups based on penicillamines, (2) thiazolidine-4-carboxylic acid, (3) oxazolones, (4) derivatives of glycine, and a miscellaneous group of intermediate and associated products. All the compounds were found to possess poor antibacterial activity *in vitro* when compared with penicillin. Those penicillamine esters which were found to possess antibacterial activity were inactivated by the presence of 10 per cent. of blood or serum, and their mode of action is unrelated to that of penicillin. All the active compounds, when given intraperitoneally, were acutely toxic in small doses to mice, and none possess chemotherapeutic value.

S. L. W.

Penicillin, Cytochemical Action of. J. Dufrenoy and R. Pratt (*J. Bact.*, 1948, 55, 525.) Agar plates seeded with *Staphylococcus aureus*, *Bacillus subtilis* or *Proteus vulgaris* were incubated until the organisms reached the logarithmic phase of growth. Aqueous solutions of penicillin in the usual assay cylinders were then allowed to diffuse through the medium for a period too short to permit the development of clear zones. On flooding the plates with appropriate reagents inhibition zones were immediately visible. Application of Pappenheim's stain, which differentiates between ribo- and desoxyribo-nucleic acid derivatives, showed that the inhibition zones were free from ribo-nucleic acid. Application of redox indicators showed that the inhibition zones had low dehydrogenase activity. Cobra venom behaved similarly to penicillin, and since it is known to interfere with sulphhydryl groups on which dehydrogenase activity depends it is possible that the same mechanism is involved in the action of penicillin.

H. T. B.

Penicillin Effectiveness, Enhancement by Traces of Cobalt. L. A. Strait, J. Dufrenoy and R. Pratt, with V. Lamb. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 133.) The addition of traces of hydrated cobalt chloride to the agar medium used in the assay of penicillin by the cylinder-plate method caused a pronounced increase in the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on the plates infected with certain bacteria. Concentrations ranging from 0.1 to 10 p.p.m. were used and the effectiveness of the penicillin solution in producing inhibition zones was increased 4 to 8 times using *Staphylococcus aureus*. Similar increases in the bacteriostatic properties of penicillin on *Escherichia coli*, *Bacillus subtilis* and *Proteus vulgaris* were observed, *P. vulgaris* being especially sensitive to traces of cobalt. The presence of traces of cobalt also reduced the threshold concentration of penicillin detectable by the cylinder-plate method to one-third or less.

L. H. P.

Vitamins, Antagonistic Action of, Towards Histamine. R. Lecoq, P. Chauchard, and H. Mazoué. (*C. R. Acad. Sci., Paris*, 1948, 227, 1264.) Repeated administration of histamine produces a chronic state of nervous chronaxia which may be relieved by the subcutaneous administration of normal doses of vitamins A, B, C, nicotinamide or rutin. Of these, only vitamins C and D, and rutin, all of which have an antagonistic action towards acetylcholine, can suppress completely the chronaxic effects of anaphylactic shock. While vitamin A and nicotinamide neutralise the effects of histamine but not of acetylcholine, on the other hand vitamin H and inositol have a neutralising effect only towards acetylcholine.

G. M.

BIOCHEMICAL ANALYSIS

Amino-acids, Ninhydrin Reagent in Determination of, by Paper Chromatography. A. J. Landua and J. Awapara. (*Science*, 1949, 109, 385.) The modified ninhydrin reagent of Awapara (*J. biol. Chem.*, 1949, in the press) consisting of a 2 per cent. ninhydrin solution in methyl cellosolve water at pH 5 (citrate buffer) and containing stannous chloride, was used to develop amino-acid spots on a paper chromatogram. The spots were cut from the chromatogram and the colour intensity was measured using a Beckman spectrophotometer at 570 m μ . A table is given showing density readings obtained at various concentrations for solutions of glutamic acid, aspartic acid, glycine and alanine; in all cases the colour density was a straight line function of the concentration. The greatest source of error in this procedure was the filter paper which gave blank readings ranging from 0.070 to 0.100; this range of 0.030 units indicated an error of about 7 per cent. on a determination of 10 μ g. of amino nitrogen. Analyses carried out on the same sheet of paper could, however, be reproduced with variations of about 1 to 2 per cent. In this connection the best results were obtained when an aliquot of the coloured solution was removed from the tube and made up to a convenient volume, thus avoiding the difficult removal of all the colour on the filter paper remaining in the tube. The amount of colour adsorbed on the filter paper was constant.

R. E. S.

Insulin, Determination of Protein in. H. Cordebard and J. Schneider. (*Ann. Pharm. franc.*, 1949, 6, 542.) To 10 ml. of a solution, corresponding to 100 I.U., 1 ml. of 5 per cent. solution of sodium tungstate is added, then 1 ml. of N sulphuric acid containing 20 per cent. of sodium sulphate. The mixture is centrifuged, and the residue, after washing, is heated for 15 minutes on the water bath with 2 ml. of N potassium dichromate and 5 ml. of concentrated sulphuric acid. After cooling and dilution to 150 ml., potassium iodide is added and the mixture is titrated with 0.1N sodium thiosulphate. In calculating the percentage of insulin protein, 1 ml. of 0.1N solution is taken as equivalent to 0.75 mg. of insulin.

G. M.

Subtilin: Microbiological Assay. R. D. Housewright, R. J. Henry and S. Berkman. (*J. Bact.*, 1948, 55, 545.) Details are given of a filter-paper disc method for the assay of subtilin using *Bacillus cereus* as the test organism. The medium used consisted of Difco peptone, beef extract, yeast extract and agar, and it was found necessary to add 2 per cent. of sodium chloride and to adjust the pH to 6.4 with hydrochloric acid. The method can also be used for the assay of subtilin in certain body fluids, consistent results being obtained in the presence of more than 10 per cent.

of blood and more than 12 per cent. of serum. With the procedure described the error of assay is approximately ± 5 to 10 per cent., depending on the number of dilutions of the unknown falling within the range of the standard curve.

H. T. B.

CHEMOTHERAPY

Oestrogens related to Triphenylethylene. W. Tadros, K. Farahat and J. M. Robson. (*J. chem. Soc.*, 1949, 439.) A number of new triphenylethylene derivatives have been prepared and their oestrogenic activity has been studied by the method of Robson (*Quart. J. exp. Physiol.*, 1938, 28, 195). The compounds were prepared by addition of different *pp'*-disubstituted benzophenones to an ethereal solution of *p*-chlorobenzylmagnesium chloride and decomposition of the product with aqueous ammonium chloride, when the corresponding carbinols were obtained. Dehydration of the carbinols gave the triphenylethylenes. The compounds were dissolved in olive oil and the oestrogenic activity was examined on injection subcutaneously and on administration orally by a stomach tube into groups of 5 ovariectomised mice.

TIME REQUIRED FOR OESTROGENIC ACTIVITY TO FALL TO HALF

No.	Substance	Dose (μ g)	Time (days)
1	1 : 1-Diphenyl-2- <i>p</i> -bromophenylethylene	1000	2
2	2-Bromo-1 : 1-diphenyl-2- <i>p</i> -bromophenylethylene	1000	56
3	2-Bromo-1 : 1-diphenyl-2- <i>p</i> -chlorophenylethylene	100	11
4	Bromotri- <i>p</i> -chlorophenylethylene	5000	5
5	2-Bromo-2- <i>p</i> -chlorophenyl-1 : 1-di- <i>p</i> -bromophenylethylene	5000	10
6	2-Bromo-2- <i>p</i> -chlorophenyl-1 : 1-di- <i>p</i> -iodophenylethylene	5000	10
7	2- <i>p</i> -chlorophenyl-1 : 1-di- <i>p</i> -methoxyphenylethylene	1000	3
8	2-Bromo-2- <i>p</i> -chlorophenyl-1 : 1-di- <i>p</i> -methoxyphenylethylene	100	5
	" " " "	10*	slight
	" " " "	100*	12
9	2- <i>p</i> -Chlorophenyl-1 : 1-di- <i>p</i> -ethoxyphenylethylene	1000	slight
10	2-Bromo-2- <i>p</i> -chlorophenyl-1 : 1-di- <i>p</i> -ethoxyphenylethylene	100	3
	" " " "	10*	3
	" " " "	100*	16
11	2-Bromo-2-phenyl-1 : 1-di- <i>p</i> -ethoxyphenylethylene	10*	7
	" " " "	50*	21
	" " " "	500*	38

* Compounds in solution in olive oil administered orally by a stomach tube into groups of 5 ovariectomised mice. In all other cases compounds in solution in olive oil were injected subcutaneously into groups of 5 ovariectomised mice.

The time required for the oestrogenic activity of compounds 8 and 10 to fall to half was shorter than that of the corresponding compounds with a phenyl group only on the ethylene 2-carbon atom. At the dose level employed the compounds 4, 5, and 6 were active in contrast to the corresponding compounds with no halogen in the phenyl group, which were inactive.

R. E. S.

Oestrogens, Synthetic, related to Triphenylethylene. W. Tadros. (*J. chem. Soc.*, 1949, 442.) Heating di-*p*-benzyloxyphenylbenzylcarbinol and 2-phenyl-1 : 1-di-*p*-isopropoxyphenylethylene *in vacuo* in the presence of a trace of sulphuric acid, or refluxing the solution in acetic acid in the presence of sulphuric or hydrobromic acids yielded 2-phenyl-1 : 1-di-*p*-hydroxyphenylethylene. 2-Bromo-2-phenyl-1 : 1-di-*p*-hydroxyphenylethylene and its diacetate (which can be obtained by bromination of 2-phenyl-1 : 1-di-*p*-acetoxyphenylethylene) were found to be highly active oestrogenic compounds when injected subcutaneously into ovariectomised mice. A number

CHEMOTHERAPY

of new triphenylchloroethylenes were prepared by chlorination of the corresponding ethylenes; di-*p*-ethylthiophenylbenzylcarbinol and di-*p*-n-propylthiophenylbenzylmagnesium chloride were obtained by adding the appropriate ketone to the ethereal solution of benzylmagnesium chloride.

R. E. S.

PHARMACOGNOSY

Aloes, Preparation of Powdered. I. W. Spoon and W. M. Sessler. (*Pharm. Weekbl.* 1949, 84, 241.) The authors give a description of the method used in the Dutch Antilles (including Curaçao) for the preparation of aloes. The boiling of the juice is carried out in copper kettles, but it is not possible to control the process properly, so that some decomposition probably occurs. A sample of the sap, preserved with chloroform, and transported in cold storage, was obtained. The liquid showed a considerable amount of sediment, which redissolved on warming; the density was 1.0911, and the solid content 25.5 per cent. The liquid was spray dried at a temperature of 71° to 74°C. The powder obtained contained 2.4 per cent. of water, was lighter in colour, and more soluble than the ordinary block aloes, while it satisfied all official requirements. It was hygroscopic, but did not cake unless the humidity of the atmosphere exceeded 70 per cent.

G. M.

PHARMACOLOGY AND THERAPEUTICS

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S. L. W.

of blood and more than 12 per cent. of serum. With the procedure described the error of assay is approximately ± 5 to 10 per cent., depending on the number of dilutions of the unknown falling within the range of the standard curve.

H. T. B.

CHEMOTHERAPY

Œstrogens related to Triphenylethylene. W. Tadros, K. Farahat and J. M. Robson. (*J. chem. Soc.*, 1949, 439.) A number of new triphenylethylene derivatives have been prepared and their œstrogenic activity has been studied by the method of Robson (*Quart. J. exp. Physiol.*, 1938, 28, 195). The compounds were prepared by addition of different *pp'*-disubstituted benzophenones to an ethereal solution of *p*-chlorobenzylmagnesium chloride and decomposition of the product with aqueous ammonium chloride, when the corresponding carbinols were obtained. Dehydration of the carbinols gave the triphenylethylenes. The compounds were dissolved in olive oil and the œstrogenic activity was examined on injection subcutaneously and on administration orally by a stomach tube into groups of 5 ovariectomised mice.

TIME REQUIRED FOR ŒSTROGENIC ACTIVITY TO FALL TO HALF

No	Substance	Dose (μ g)	Time (days)
1	1:1-Diphenyl-2- <i>p</i> -bromophenylethylene	1000	2
2	2-Bromo-1:1-diphenyl-2- <i>p</i> -bromophenylethylene	1000	56
3	2-Bromo-1:1-diphenyl-2- <i>p</i> -chlorophenylethylene	100	11
4	Bromotri- <i>p</i> -chlorophenylethylene	5000	5
5	2-Bromo-2- <i>p</i> -chlorophenyl-1:1-di- <i>p</i> -bromophenylethylene	5000	10
6	2-Bromo-2- <i>p</i> -chlorophenyl-1:1-di- <i>p</i> -iodophenylethylene	5000	10
7	2- <i>p</i> -chlorophenyl-1:1-di- <i>p</i> -methoxyphenylethylene	1000	3
8	2-Bromo-2- <i>p</i> -chlorophenyl-1:1-di- <i>p</i> -methoxyphenylethylene	100	5
	" " " "	10*	slight
	" " " "	100*	12
9	2- <i>p</i> -Chlorophenyl-1:1-di- <i>p</i> -ethoxyphenylethylene	1000	slight
10	2-Bromo-2- <i>p</i> -chlorophenyl-1:1-di- <i>p</i> -ethoxyphenylethylene	100	3
	" " " "	10*	3
	" " " "	100*	16
11	2-Bromo-2-phenyl-1:1-di- <i>p</i> -ethoxyphenylethylene	10*	7
	" " " "	50*	21
	" " " "	500*	38

* Compounds in solution in olive oil administered orally by a stomach tube into groups of 5 ovariectomised mice. In all other cases compounds in solution in olive oil were injected subcutaneously into groups of 5 ovariectomised mice.

The time required for the œstrogenic activity of compounds 8 and 10 to fall to half was shorter than that of the corresponding compounds with a phenyl group only on the ethylene 2-carbon atom. At the dose level employed the compounds 4, 5, and 6 were active in contrast to the corresponding compounds with no halogen in the phenyl group, which were inactive.

R. E. S

Œstrogens, Synthetic, related to Triphenylethylene. W. Tadros. (*J. chem. Soc.*, 1949, 442.) Heating di-*p*-benzyloxyphenylbenzylcarbinol and 2-phenyl-1:1-di-*p*-isopropoxyphenylethylene *in vacuo* in the presence of a trace of sulphuric acid, or refluxing the solution in acetic acid in the presence of sulphuric or hydrobromic acids yielded 2-phenyl-1:1-di-*p*-hydroxyphenylethylene. 2-Bromo-2-phenyl-1:1-di-*p*-hydroxyphenylethylene and its diacetate (which can be obtained by bromination of 2-phenyl-1:1-di-*p*-acetoxyphenylethylene) were found to be highly active œstrogenic compounds when injected subcutaneously into ovariectomised mice. A number

CHEMOTHERAPY

of new triphenylchloroethylenes were prepared by chlorination of the corresponding ethylenes; di-*p*-ethylthiophenylbenzylcarbinol and di-*p*-n-propylthiophenylbenzylmagnesium chloride were obtained by adding the appropriate ketone to the ethereal solution of benzylmagnesium chloride.

R. E. S.

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G. M.

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S. L. W.

Diparcol in Parkinsonism. R. S. Duff. (*Brit. med. J.*, 1949, 1, 613.) Diparcol, a proprietary brand of diethylaminoethyl-N-thio-diphenylamine hydrochloride, was employed in the treatment of 8 cases of post-encephalitic chronic Parkinsonism of varying degrees of severity. Starting with small doses the amount was gradually increased up to 1 g. daily by mouth, and this dose continued for a period of 10 weeks until significant benefit was obtained, after which the dose was varied by small amounts to suit the requirements of the patient. It was found that treatment with two 0.25 g. tablets on awakening, followed by one tablet at noon, and one at about 5 p.m., brought the greatest reduction of rigidity. Diparcol seemed to offer some advantages over the tropine series of alkaloids (exemplified by stramonium), and all the patients derived a little benefit as compared with previous treatment. Improvement in the feeling of well-being was noteworthy in several of the patients. Oculogyric crises were not significantly reduced. Hypersalivation was not well controlled in 4 of the cases, in which the addition of small doses of belladonna became necessary. Undesirable effects in some patients included faintness, paræsthesiæ, transient blurring of vision, undue drowsiness. There was a tendency for the white cell count to fall to 4000 per c.mm. during the initial weeks with a subsequent rise to former levels. A method of recording and evaluating the results of treatment of patients with chronic disorders of locomotion is described.

S. L. W.

Di-isopropyl Fluorophosphate (D.F.P.) in Surgery. J. P. Quilliam and T. A. Quilliam. (*Lancet*, 1949, 256, 603.) The use in post-operative paralytic ileus is illustrated in 12 successfully treated cases. It should be used as a supplement to other measures, 2 to 4 mg. being given by intramuscular injection, and the dose repeated after 12 to 24 hours if defæcation has not occurred. If the case is very urgent a maximum of 2 mg. may be given 4 hours after the first dose. It is more certain in its action on paralytic ileus than neostigmine and does not give rise to the unpleasant and sometimes dangerous side-effects which may attend the use of posterior pituitary extract, while the beneficial effects seem to last longer than those of either of these drugs. It was also used successfully in 5 cases of post-operative abdominal distension and 4 cases of intestinal obstruction and in post-operative retention of urine. As a simple biological test to assess potency a small measured volume is instilled into the right conjunctival sac of a rabbit, and a similar volume of a standard solution into the left sac. A comparison of the mean pupil diameters under constant conditions of illumination made at 10 minute intervals during the hour following administration will indicate the potency of the sample against the standard. There was little, if any, loss in potency of an arachis oil solution after storage for up to 2 years at room temperature.

S. L. W.

Hexa-ethyltetraphosphate, Toxicology and Pharmacology of. S. Forssling. (*Acta Pharmacol. Toxicol.*, 1948; 4, 143.) Comparative toxicity tests on hexa-ethyltetraphosphate with and without atropine prophylaxis were carried out on the mouse, rat, guinea-pig, rabbit, and cat. The administration was subcutaneous, also percutaneous for the rat and guinea-pig, and oral for the rat. The inhibitive action of hexa-ethyltetraphosphate on acetylcholinesterase was demonstrated by recording the blood pressure. The LD50 by subcutaneous administration was: for mouse 0.9, rat 0.7, guinea-pig 2.2, rabbit 2.0-2.5 and cat 2.5-3.0 mg./kg. In the rat the relation between LD50 by subcutaneous, oral and percutaneous administration

was 1:2.5:35. Certain animals, particularly mice are more protected than others with atropine prophylaxis. The mortality is hardly reduced in the rabbit although the survival time is considerably prolonged. The guinea-pig and rat, after atropine, tolerate a 3-fold increase in the dose of hexa-ethyltetraphosphate. With di-isopropylfluorophosphate and hexa-ethyltetraphosphate the animals investigated seemed to be better protected by magnesium sulphate and atropine than by tropine alone; physostigmine in large doses after a small dose of atropine gave the best protection. The inhibition of acetylcholine-esterase is essential for the toxicity: in the cat this inhibition can be demonstrated *in vivo* by recording the momentary decrease in blood pressure after an intravenous injection of acetylcholine-esterase before and after the administration of hexa-ethyltetraphosphate. The nicotine but not the muscarine activity in hexa-ethyltetraphosphate poisoning is eliminated in the cat by urethane narcosis, resulting in a considerable increase in tolerance.

R. E. S.

Paludrine, Some Pharmacological Actions of. J. R. Vane. (*Brit. J. Pharmacol*, 1949, 4, 14.) The LD₅₀ for acute intravenous toxicity in mice was 22 mg./kg., this figure being calculated after observing the mice for 72 hours after the injection; the simultaneous injection of neostigmine increased the immediate toxic effect. Paludrine antagonised the action of acetylcholine or of vagal stimulation; it inhibited the contractions of the isolated frog rectus muscle and guinea-pig ileum and abolished the action of acetylcholine on isolated rabbit auricles; it decreased the response of the cat's intestine to vagal stimulation, and caused depression of respiration in the rabbit. It lengthened the refractory period of auricular tissue and had a curariform action on the cat sciatic-gastrocnemius, the rat phrenic nerve-diaphragm, and the perfused superior cervical ganglion preparations. Paludrine caused vasodilatation of the perfused dog hind leg, and in the cat, this dilation was reduced by antihistamine agents, suggesting that paludrine might release histamine from the tissues. If this is so it presents a difficulty in assessing the relationship between paludrine and histamine: whereas paludrine inhibits gastric secretion evoked by histamine, and reduces the response of isolated guinea-pig ileum to histamine, it potentiates the action of histamine on guinea-pig lungs. On the other hand, antihistamine agents, which abolish the effect of histamine in most hormones but potentiate the gastric secretion caused by histamine, reduced the action of paludrine on the systemic vessels.

S. L. W.

Penicillin, Radioactive, Investigations with. P. D. Cooper and D. Rowley. (*Nature*, 1949, 163, 480.) A penicillin uptake was detected by counting thin films of bacteria. Bacteria were suspended under various conditions in radioactive penicillin solutions, centrifuged, washed and the radioactivity on the bacteria measured directly by Geiger counter, the uptake being expressed as units of penicillin per g. of dry weight of the cells. The penicillin uptake was small, from a maximum of 40 units /g. of dry weight to the smallest detectable amount of 0.5 units /g. depending on the strain of the organism and on the conditions of experiment. It was found that in the range of 0.0 to 0.5 units/ml. much more penicillin was fixed by sensitive than by resistant bacteria, there being a direct correlation between the sensitivity of an organism and the amount of penicillin attached to it. The uptake increased when growth occurred in the presence of penicillin although by cooling, or with dead cells, there was still a rapid

but smaller uptake. The penicillin was strongly and irreversibly attached to the cells and could not be removed by washing or by incubation for 30 minutes with 0.1 per cent. of cetyltrimethylammonium bromide or "Aerosol OT," 5 per cent. of phenol, 1 per cent. of cysteine, 1 per cent. of sodium hydroxide, N hydrochloric acid, 1 per cent. or glucose or with ordinary penicillin (200 u./ml.), although it was removed by heating for 5 days at 60°C. in water. The uptake of penicillin was not prevented by pre-treatment with 3 per cent. of formalin, 0.1 per cent. of cetyltrimethylammonium bromide, 0.1 per cent. of euflavine, N hydrochloric acid, or by autoclaving, but heating for 5 days at 60°C. in water or pre-treatment with penicillin or acetic anhydride completely prevented any uptake. It is clear that rapid growth exposes more centres in the bacteria with which the penicillin can react.

R. E. S.

Penicillin: Single Daily Dose in Treatment of Pneumonia. W. Weiss and I. Steinberg. (*Amer. J. med. Sci.*, 1949, 217, 86.) Thirty consecutive pneumonia cases were treated by a single daily injection of 300,000 units of penicillin G in aqueous solution. Treatment was continued for from 4 to 13 days, usually from 5 to 7 days. In 18 cases, the temperature dropped to normal by crisis within 12 to 36 hours. In 9 patients the temperature fell by lysis, and longer treatment, up to 13 doses, was necessary. Of the remaining 3 patients, in one the condition was due to *Streptococcus viridans* and failed to respond; the other 2 terminated fatally, although in both cases the lobe involved was sterile. The results are considered to be at least as good as reported by others using smaller doses at more frequent intervals.

H. T. B.

Stramonium Powders, Investigations into the Alkaloidal Content of Smoke from. P. Terp. (*Acta Pharmacol. Toxicol.*, 1949, 4, 135.) The amount of tropa-alkaloid present in the smoke from burned stramonium powder has been investigated. The smoke was absorbed in a series of absorption bottles containing dilute hydrochloric acid and the final estimation of tropa-alkaloids was made on a biological basis, using Pulewka's method, (*Arch. exp. Path. Pharmacol.*, 1932, 168, 307) by measuring the pupil dilation of the mouse's eye. This gave an indication of how much biological activity present in the starting material had been recovered in the smoke, but not how much was due to atropine and hyoscyamine respectively; nor did it give information as to whether the burning caused racemisation. It was found that 8 to 11 per cent. of the amount of alkaloid in the starting material (as atropine sulphate) was present in the smoke. Less than 1 per cent. remained in the ash. Experiments on mice showed that the greater part of the alkaloid entering the respiratory organs with the smoke, was absorbed.

R. E. S.

Streptomycin in Non-tuberculous Infections. P. H. Buxton, R. D. Simon and F. R. Selbie. (*Lancet*, 1949, 256, 729.) This is a report on the treatment of 67 cases of infection by organisms insensitive to the sulphonamides and penicillin but sensitive to streptomycin. These included 44 cases of urinary infection, chiefly by *Pseudomonas pyocyanea* and *Bacterium coli*, 14 cases of wound infection, mainly by *Staphylococcus pyogenes*, and 9 miscellaneous infections. A dosage of 3 g. of streptomycin daily for 4 days in urinary infections due to a susceptible organism is likely to cure about one-third of the cases, provided there is no mechanical obstruction to drainage; all patients in whom the infecting organisms were eliminated had sterile urine within 72 hours, and usually within 24 hours,

of starting treatment, any organisms still present after 72 hours showing greatly reduced sensitivity to streptomycin. In the wound infections due to staphylococci the results were promising, of 14 patients treated 10 were cured and 2 improved. The usual dosage was 4 g. daily for 2 days, followed by 2 g. daily. In superficial infections a powder consisting of streptomycin 1 g., penicillin 45,000 units, and suphathiazole 9 g. was also given locally by insufflation every 3 or 4 hours to give a total daily dosage equivalent to 1 g. of streptomycin. If no improvement occurs in these cases within a week of starting streptomycin, further treatment by surgery to eliminate inaccessible foci of infection and provide adequate drainage should be considered. Side-reactions occurred in less than one-third of the cases, and in only 3, where there was severe vestibular disturbance, were they of serious significance.

S. L. W.

Strychnos lucida, Alkaloids of. F. H. Shaw and I. S. de la Landa. (*Austral. J. exp. Biol.*, 1948, 26, 199.) *Strychnos lucida* is an Australian *Strychnos* species closely related to *Strychnos nux vomica*. In the present investigation the presence of strychnine (approximately 0.3 per cent.) and brucine (1.5 to 2.4 per cent.) in the seed has been confirmed by their isolation, and their probable presence in the leaf and bark indicated by assay data. In addition, non-strychnine-brucine alkaloid fractions have been isolated from the seed and leaf. These have been tentatively called lucidine-S and lucidine-L respectively. These alkaloid fractions are both amorphous and have similar solubilities to brucine. Both were isolated in small quantities from the plant by a method which is described, and both appear to be identical, though owing to the inability to isolate the bases or their salts in a crystalline condition it is not known whether they are homogeneous or not. The pharmacological investigation of lucidine-S was restricted to toxicity tests on mice and rabbits, which indicate that the intravenous LD₅₀ is greater than 250 mg./kg. and 60 mg./kg. for these animals respectively. Toxic doses in mice produced strychnine-like convulsions. The toxicity of lucidine-L is of the same order as that of lucidine-S and toxic doses produced similar strychnine-like convulsions; it does not show any marked physiological activity. In anaesthetised cats and dogs (10 mg./kg.) it produces a fall in blood pressure similar to that produced by brucine.

S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

2:4-Dichlorophenoxyacetic Acid, Differential Effects of, on Aerobic, Anaerobic and Facultative Anaerobic Micro-organisms. A. Worth, Jr., and A. M. McCabe. (*Science*, 1948, 108, 16.) This substance is biologically recognised as an auxin or growth-regulating substance. It is known to have bacteriostatic and bactericidal properties, and to inhibit growth of typically aerobic seeds, such as barley seeds, but not the growth of seeds like those of rice, which are able to germinate in the absence of oxygen. To demonstrate the effects of varying concentrations of 2:4-dichlorophenoxyacetic acid on the growth of micro-organisms which differ in their utilisation of oxygen, 10 organisms were chosen. The aerobes were *Rhizobium trifolii*, *R. phaseoli*, *R. japonicum* and *Azotobacter chroococcus*; the anaerobes were *Clostridium welchii*, *Cl. botulinum* and *Cl. tetani*; and the facultative anaerobes were

[Continued on page 733

BOOK REVIEWS

MATERIA MEDICA. PHARMACY, PHARMACOLOGY AND THERAPEUTICS, by *W. Hale-White*, 28th Edition, revised by *A. H. Dowd*. Pp. 507 with Appendix and Index. J. & A. Churchill, Ltd., London 1949. 16s. 0d.

There are many books which attempt to present the subjects of materia medica, pharmacology and therapeutics in a form acceptable to the student and to the medical practitioner. The most frequent criticism of such publications is that they either provide too much or too little information. Probably the chief fault of a book such as Hale-White's "Materia Medica" is that the concise nature of the text does not afford a fuller presentation of the available evidence on the pharmacological action of drugs. This does not permit discussion of some aspects of the subject where it is perhaps desirable to distinguish between opinions arising from conjecture and statements based on fact. In conformity with the traditional nature of the book there is a comprehensive list of the drugs and preparations described in the British Pharmacopœia. For the student of pharmacy this may be a desirable feature, but it is doubtful whether the medical student can derive much benefit from such an array of names except as a dictionary of reference. He might well be puzzled regarding the necessity for a strong tincture and a weak tincture of ginger; a strong and weak solution of ammonium acetate; a liquid extract, two infusions and a tincture of senega, especially when in the latter instance the text indicates that they are only occasionally used as expectorants. A text-book which is revised in the course of over half a century has provided on the average a revision every second year, requires no further comment on its popularity. The 28th edition of this book incorporates all the numerous changes in the drugs and preparations resulting from the appearance of the British Pharmacopœia of 1948.

ANDREW WILSON.

GRUNDLAGEN DER PHARMAKOLOGIE, by *K. W. Merz*. Pp. 272 with Index. Wissenschaftliche Verlagsgesellschaft M.B.H. Stuttgart, 1948.

Professor Merz, who was formerly Director of the Institute of Pharmaceutical Chemistry in the University of Königsberg, has completed the fourth edition of his text-book of Pharmacology. It is primarily intended for pharmacists, chemists and biologists, and if chemical formulæ might be regarded as an inducement to the reader, there is much to attract his attention. The book is divided into 18 chapters which permits a systematic and quite comprehensive treatment of the subject. The first three chapters consist of a general discussion of definitions, types of pharmacological action and the mechanism of drug action. In the remaining sections the action and uses of drugs are discussed in relation to the systems of the body, in much the same fashion as in Clark's "Applied Pharmacology." There is a concise description of the anatomy, and physiology, and where appropriate, of the pathology relevant to each such system. A very useful chapter deals with the toxicology of the common organic solvents and gases. On the whole the account of the pharmacological actions is sound though in some instances the author appears content to concern himself only with what happens in the frog and not in the higher species, particularly in man. It is desirable also to indicate more clearly that the parasympathomimetic drugs do not stimulate nerve endings, though their action resembles this effect. The book is well illustrated with chemical formulæ, line drawings and photographs of original

tracings. It is unfortunate that the quality of the paper is poor and does not permit a reasonable reproduction of those photographs which are intended to display characteristic clinical features. In some instances the effect is spoiled by lack of definition in which important detail is not clearly visible. For those to whom the German language presents no difficulties the book is well worth reading; others may prefer to await a suitable English translation.

ANDREW WILSON.

BENTLEY'S TEXTBOOK OF PHARMACEUTICS, by Harold Davis. Pp. xiv + 1100 and Index. Fifth Edition. Bailliere, Tindall & Cox, London. 1949. 30s. net.

While welcoming the fifth edition of Bentley and recommending it to all students of pharmacy as an essential text-book, one wonders whether it is in fact possible to produce successfully in one volume, a satisfying account of the very numerous pharmaceutical phenomena, together with their explanations, descriptions of machinery and their uses, dispensing of medicines, surgical dressings, bacteriology, immunology, and pharmaceuticals even in 1100 pages. The numerous references to the original literature without which no modern text-book is of value, help in some measure to ease the path of the earnest student, but many of the less critical students will neglect the implied advice of the authors to read widely and the curriculum does not allow much time for work in the library. Having said this, in the hope that Dr. Davis and his collaborators will feel only partially satisfied with their efforts and from their wide experience and knowledge will be inspired in the near future to produce a series of volumes consisting of the sections of the present volume each expanded to a volume of its own, congratulations are extended to the authors for the accomplishment of a really formidable task and encouragement is offered to go even further. Such a work would help to meet the needs of critical students of whom there is an ever-increasing number.

The volume is well printed by modern British standards and is freely illustrated. Many of the illustrations are excellent but some of the photographs have not reproduced well and as sources of information the sketches on pages 489 and 490 would convey little to one not already familiar with the equipment concerned. The proof reading has been well done although the reference to chapter LXXVI on page 47 should read LXXVII. J. P. TODD.

ABSTRACTS (Continued from Page 731)

Escherichia coli, *Staphylococcus albus* and *Candida albicans*. Different media were used for different organisms, but the reaction was adjusted in all cases to pH 7.4. The concentrations tested were 2, 1, 0.2, 0.02, 0.002 and 0.0002 per cent. in 5 ml. of medium; this was poured into Petri dishes containing about 10 to 15 ml. of the corresponding medium. Each micro-organism was grown on these six concentrations, and on a control plate, the aerobes were cultivated at room-temperature, the anaerobes and facultative anaerobes were incubated at 37.5°C., the anaerobes in Brewster anaerobic jars. The amount of growth was compared with the control at intervals of 24, 48 and 72 hours. Growth of *R. phaseoli* and *R. japonicum* appeared to be inhibited after 48 hours, but some growth appeared in the lower concentrations after 72 hours: whereas *R. trifolii* and *A. chroococcus* were inhibited only temporarily by the higher concentrations, lower concentrations increased the amount of growth. No inhibitory effects were observed with the facultative anaerobic organisms or with the anaerobes.

L. H. P.

LETTERS TO THE EDITOR

The Inactivation of Penicillin by Oil of Theobroma

SIR,—During the development of penicillin formulations for human and veterinary use we have had occasion to investigate the effect of incorporating various penicillin salts in bases containing oil of theobroma. The rapid fall of potency in these preparations led us to suspect that the latter was exerting a definite inactivating effect. A series of dispersions of penicillin in oil of theobroma was accordingly prepared, using separately calcium penicillin and crystalline potassium penicillin at concentrations of approximately 10,000 I.U./g. These were assayed at time intervals after storage at room temperature and a progressive potency fall was observed. For calcium penicillin a fall of 27 per cent. was recorded after 3 months and for crystalline potassium penicillin a fall of 90 per cent. after 12 months. Further work is in progress to investigate this change in more detail but meanwhile we think it advisable to draw attention to what may be a serious incompatibility.

J. C. FLOYD.

Pharmaceutical Research and Service Laboratory,
Imperial Chemical Industries,
Blackley, Manchester, 9.

August 16th, 1949.

Vitamin B₁₂ as a 5:6-Dimethylbenziminazole Derivative

SIR,—A paper chromatogram of an acid hydrolysate of vitamin B₁₂ (*n*-butyl alcohol-acetic acid being employed as the irrigation solvent) was exposed to the light of a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter, when three blue fluorescent spots were revealed. Spectroscopic examination of eluates from these areas indicated their close chemical similarity. The compounds responsible for the fluorescent zones have accordingly been termed by us *components* α -, β -, and γ . Comparison of their ultra-violet absorption spectra with those of known heterocyclic ring systems led to their identification as derivatives of benziminazole. Spectroscopic comparison with 22 methylated benziminazoles synthesised for this purpose led to the identification of *component* γ with 5:6-dimethylbenziminazole, and of components α - and β - with 1-substituted 5:6-dimethylbenziminazoles. Moreover, both spectroscopic and chemical work has led us to the conclusion that vitamin B₁₂ itself contains one preformed benziminazole residue in the molecule. It may therefore be inferred that *components* α -, β -, and γ represent different stages of degradation of a common precursor. It is interesting to note that vitamin B₁₂ and riboflavine may thus both be regarded as derived chemically from 4:5-dimethyl-*o*-phenylenediamine, and speculation on the biogenesis of vitamin B₁₂ thus becomes possible.

Both Dr. K. Folkers and one of us (V.P.) have to-day simultaneously reported at the 1st International Congress of Biochemistry held at Cambridge the identification of hydrolytic fragments of vitamin B₁₂ with 5:6 dimethylbenziminazoles. It therefore seems desirable to place these observations on

record at this stage. Our detailed results will be submitted shortly for publication in your Journal.*

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

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August 22, 1949.

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The "Ninhydrin-Reacting" Hydrolytic Fragment of Vitamin B₁₂

SIR,—We have previously reported¹ that hydrolysis of vitamin B₁₂ with 20 per cent. hydrochloric acid at 100°C. for 6 hours followed by examination of the hydrolysate by unidimensional paper-strip chromatography, reveals the presence of one "ninhydrin-reacting" substance which could not be identified with any of the known amino-acids. Our studies have hitherto been handicapped by incomplete separation on paper chromatograms of the "ninhydrin-reacting" fragment from other products of vitamin B₁₂ hydrolysis. By using *n*-butyl alcohol-acetic acid as the irrigation solvent, however, complete separation has now been obtained. The "ninhydrin-reacting" area occupies a position well removed from zones which fluoresce under the light of a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter², and which form the subject of a separate communication (*vide infra*). Elution of the "ninhydrin-reacting" area with dilute hydrochloric acid gives a solution transparent to ultra-violet light. From this and other observations we concluded that the "ninhydrin-reacting" substance was probably an aliphatic base.

We now find that the "ninhydrin-reacting" substance and 2-aminopropanol show identical behaviour on paper chromatograms irrigated with four different solvent systems. The two substances thus have the same partition coefficients in each of these solvent systems, and it is therefore reasonable to conclude that they are identical. A final decision, however, must rest on a direct chemical comparison. Full details of this work have already been submitted for publication³.

The authors thank the Directors of The British Drug Houses, Ltd., for permission to publish these results.

Research Department,
The British Drug Houses, Ltd.,
London, N.1.
August 22, 1949.

B. ELLIS,
V. PETROW,
G. F. SNOOK.

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NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Kina-Redoxon* tablets contain quinine and vitamin C and are indicated for the prophylaxis and treatment of influenzal conditions, the common cold, coryza, etc. Sugar-coated tablets are supplied in bottles of 50 and 100.

S. L. W.

Lipo-Adrenal Cortex. (*New and Nonofficial Remedies. J. Amer. med. Ass., 1949, 139, 849.*) Lipo-adrenal cortex is an oil-soluble extract of hog suprarenal glands containing crystalline, biologically active constituents of what are considered to be 17-hydroxycorticosterone, 11-dehydro-17-hydroxycorticosterone and corticosterone, and a non-crystallisable amount of 11-dehydrocorticosterone. It is almost free from adrenaline and is assayed biologically. It has about 10 times the suprarenal cortical activity of adrenal cortex extract N.N.R. and is used for the same purposes when a more prolonged action is required. It is supplied as a sterile solution in cotton-seed oil and is administered intramuscularly in doses up to 3 ml., depending on the degree of cortical insufficiency and clinical response. It should be supplemented by the administration of sodium salts. The method of preparation is described.

G. J. K.

Parvestin* is a concentrated desiccated extract made from the small intestine of the pig, one heaped teaspoonful corresponding to about 4 ounces of the fresh intestine. It is indicated in the treatment of chronic ulcerative colitis. The dose is 1 or 2 teaspoonfuls daily, reduced to 1 teaspoonful daily as improvement occurs. If preferred, the powder may be stirred into warm beverages or mixed with food. Treatment usually requires to be continued for 4 to 8 weeks. Parvestin is supplied in 4-oz. bottles.

S. L. W.

Tyroderm* is an antibiotic cream containing 0.5 mg. of tyrothricin per g. in a water-soluble base. It is indicated in the treatment of pyodermatoses, including acne vulgaris, dermatitis and other dermatoses due to Gram-positive organisms. It may also be of value in varicose, decubital and ischaemic ulcers infected with Gram-positive organisms, and in the treatment of accessible post-surgical wounds and potentially contaminated minor burns. The cream should be applied at least once daily and may be covered with a dressing if necessary. It is supplied in 2 oz. tubes.

S. L. W.

Unden* is a brand of oestrone, available either in pellets, ointment or aqueous solution, 1 mg. of which is equivalent to 10,000 international oestrone units, or (as oestrone benzoate) in oily solution, 1 mg. of which is equivalent to approximately 10,000 oestradiol benzoate units. The ointment is rubbed thoroughly into the affected areas of skin several times a day. The packings are as follows:—pellets containing 0.01, 0.05 or 0.1 mg. in bottles of 15 or 100; ampoules (aqueous) in boxes of 5 × 1 ml., each ampoule containing 0.1 mg.; ampoules (oily) 1 ml., each containing 5 mg. of oestrone benzoate solution (oily); bottles of 10 ml., each ml. containing 1 mg. of oestrone benzoate; ointment in pots of 20 g., containing 0.5 mg./g.

S. L. W.

REVIEW ARTICLE

THE INTERNATIONAL STANDARD FOR VITAMIN D

By KATHARINE H. COWARD, D.Sc.

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THE International Standards for the determination of the different vitamins are part of the indispensable equipment of the laboratories in which this work is carried out. Though the standard materials themselves are so well known, the amount of labour which has been devoted to their creation and adoption and the infinite care which has been taken to ensure their suitability, accessibility and integrity are not so well known or appreciated. The adoption of a crystalline preparation of vitamin D₃ to serve as the International Standard for Vitamin D affords a suitable opportunity for outlining the nature of this work, the importance of which to scientific research, medical practice, the problems of nutrition, and the international exchange of ideas, results and materials is now well recognised.

In this country the Therapeutic Substances Act (1925) was passed to deal with the administrative control of those substances of therapeutic value, the potency of which could not be adequately tested by chemical and physical means. Such substances are of many different kinds and, since their potency could only be determined by biological tests carried out in strict comparison with a standard preparation, the necessity for these was fully recognised; and, since the same materials and medicaments are used in many lands, it was obvious that the standards for their measurement should have an International standing and application. International Standards for the assay of antitoxins, insulin, pituitary (posterior lobe) powder and digitalis had been established before the similar work for vitamins was begun. Very few firms, however, were equipped to carry out the necessary biological determinations on their products of therapeutic application. Accordingly, to meet the demand for such tests, the Pharmaceutical Society of Great Britain established its Pharmacological Laboratory in January, 1926. Within a very few months requests for the determination of vitamin D in cod-liver oils were made by firms who realised that the vitamin D content of these oils varied within wide limits and who knew also that it was possible to extract the vitamin D of the oil without detection of the loss, and the Pharmaceutical Society after consulting the Accessory Food Factors Committee of the Medical Research Council set up a Vitamin-testing Department within the Pharmacological Department. The writer of this review was appointed to take charge of it and in January, 1927, the available laboratories had been put into a condition suitable for the satisfactory performance of the tests. Actually, the task of devising a reasonable uniform diet for albino rats—on which they would grow, reproduce and lactate and, moreover, would yield a progeny having minimum reserves of vitamins A and D, so that they could be used at an early age for the comparative tests—proved

to be of paramount importance and has so remained throughout the years. The account of the work in this field is described elsewhere¹. Suffice to say here that the diet found to be satisfactory by 1928 has, with only minor modifications such as the exigencies of the times demanded, proved to be satisfactory up to the present time.

Up to the time of setting up this laboratory, tests had been really only qualitative, and the relative vitamin potencies of foods indicated by the signs +, ++, +++, or +++, according to the amounts, roughly, of the different foods required for bringing a certain amount of reaction which was known to be extremely variable and which could only be controlled by comparative tests in relation to Standard preparations; and it was not until 1931 that the extremely important step of establishing International Standards for the assay of vitamins A, B₁, C and D was taken by the Permanent Standards Commission of the Health Organisation of the League of Nations².

By 1926 certain criteria for measuring the response of an animal to vitamin D were already known and considered to be adaptable for use as the basis for quantitative assay; of these, the ash content of the bone (generally expressed as the percentage ash of the dry, fat-extracted bone) had been used in various laboratories for comparing different cod-liver oils; and the "line test" had been used for the same purpose and, particularly in Steenbock's laboratory, for following the antirachitic activation of foods, food-constituents and cholesterol by irradiation with ultra-violet rays. In his laboratory the amount of healing, indicated by the width of the line of calcification, was assessed in five grades, 0, +, ++, +++, +++, the last indicating complete healing. It was decided to adopt the "line test" in our own laboratory as it was quicker and less laborious than the "ash content of the bone" method. While we were raising our colony of rats to the right condition for these tests, we used litters drawn partly from our own colony and partly from those of other laboratories. The inconvenience of this was offset by the value of the information it gave on how greatly rats obtained from different colonies and subjected to the same treatment may differ in their response to the same dose of vitamin D. Ample evidence was also obtained of the difference in response of different litters from the same colony. All this afforded proof—if any were needed—of the necessity to provide for the comparative tests some stable preparation of vitamin D which could be used as a standard of reference and tested simultaneously with every vitamin D determination.

About this time Rosenheim and Webster³ were irradiating ergosterol with ultra-violet light and getting intensely active antirachitic material as shown by tests on rats very similar to the line test, the width of the line of healing in their experiments being measured by X-ray examination of the bones. They had determined that a certain degree of activity could be produced in a solution of ergosterol by irradiating it for a certain time, that further irradiation for some time did not increase or decrease the activity but that finally the activity did fall to zero. They were therefore able to undertake to make a solution of activated ergosterol, and by

using the same conditions of irradiation, strength of solution, etc., they could reasonably expect to make a similar solution of equal potency at any future time. Such a preparation would be very nearly ideal for a standard of reference, the only point left to be determined being the stability of the preparation. This could only be done in the course of time or by expedited experiments in which a solution was subjected to more stringent conditions of temperature, etc., than it was likely to encounter during its normal use. Such experiments were carried out and no loss of activity found¹. In February, 1927, a particular sample of irradiated ergosterol in olive oil, containing the equivalent of 0.0001 mg. of the original ergosterol in 1.0 mg. of oil, was prepared by Mr. T. A. Webster of the Rosenheim and Webster team at the National Institute for Medical Research, London and adopted by the Pharmaceutical Society as its standard of reference. The unit of activity adopted was that contained in 1 mg. of this oily solution. This was the first standard of reference for any vitamin to be adopted in any country².

The first way in which the standard was used was generally to give to three rats of a litter daily doses of, say, 0.00001, 0.00002, and 0.00005 mg. of original sterol (i.e., 0.1, 0.2, 0.5 units) respectively, and to three other rats of the same litter doses of the oil under test in the same proportions. Comparisons were then made between the responses of rats on corresponding doses, and an estimate of potency made by the consideration of all the possible comparisons without the litter. Several litters were used in exactly the same way and an average of the several estimates calculated.

Later, in 1931, Dyer³ worked out a method of making the estimation more quantitative. He selected a series of cut and stained bones of rats which had been used in "line tests" which showed graded stages in healing, the widths of the lines chosen being as nearly proportional to the figures 0-6 as could be judged by the naked eye, 0 representing no healing and 6, complete healing. Thus it became possible to assign a numerical value to the healing of each rat and then total and average the healing of any number of rats all of which had received the same dose. He then constructed a curve of response to graded doses of vitamin D. 15 litters of 7 rats each were prepared in the usual way. In each litter different rats received the following daily doses of vitamin D:—no dose, 0.0625, 0.125, 0.250, 0.5, 1.0, 2.0 units. The responses of the 15 rats receiving each dose were averaged and plotted against the dose given. The curve of response was used in future tests to compare the average response of a group of rats receiving one dose of, say, cod-liver oil with that of another group receiving one dose of the standard, by finding the abscissa of the curve corresponding with the healing of each group and calculating the potency from the respective abscissae. Further work, however, showed that the curve of response to vitamin D was logarithmic in shape, whether the ash content of the bone or the line of healing was the criterion used. The slope varied from time to time and, accordingly the method of testing 2 doses of Standard against 2 doses of test substance was adopted, the average slope of the 2 curves thus obtained in each

unit of vitamin D, the activity of 1 mg. of the International Standard solution of irradiated ergosterol.

It was hoped at that time that a crystalline substance more stable than the solution of irradiated ergosterol was expected to be would soon become available. Bourdillon in this country, Windaus in Germany and Reerink and van Wijk in Holland had each prepared a crystallised substance which they thought was pure vitamin D. The three substances, however, differed in certain physical properties and each worker realised that his product was not pure. Callow purified Bourdillon's preparation by treating it with 3-5-dinitrobenzoyl chloride in pyridine and crystallising the product from acetone. Close examination revealed the fact that the product consisted of two kinds of crystals, one yellow and the other orange. Callow separated them into two groups by means of a pin and obtained 50 mg. of the yellow and 40 mg. of the orange form. He hydrolysed each and found one to be inactive biologically and the other active. He gave the name pyrocalciferol to the inactive form and retained the original name of the mixture, calciferol, for the active substance, which had about twice the antirachitic activity of the original. Meanwhile Windaus showed that his original product (which he now called D_1), really consisted of a molecular compound of lumisterol and a substance which he called D_2 . This he sent to Hampstead for comparison with Bourdillon's calciferol and it proved to have the same potency as that substance and the same physical properties. Windaus and Bourdillon concluded that they had at last arrived at the same substance. It has since been called calciferol or vitamin D_2 . It is now made on a small manufacturing scale and has been available for some years to replace the standard preparation of irradiated ergosterol, as recommended by the Second International Conference on Vitamin Standardisation. This has, however, never been necessary except on one occasion during the War when the dilution of the Standard was made with an oil which rapidly inactivated it. Reports from various workers that the standard did not appear to have its usual activity resulted in an immediate comparison between that issue and the remains of previous issues in several laboratories. It showed beyond question that that issue was not "up to Standard" and a solution of pure calciferol was made up with a carefully chosen oil and compared by several workers with a fresh dilution of the standard made with the fresh oil. The two solutions proved to be of equal potency and the activity of the original solution has never since been doubted.

The Second International Conference on Vitamin Standardisation^s held by the Permanent Commission on Biological Standardisation of the League of Nations in London, 1934, recommended that the Standard Solution of irradiated ergosterol prepared at the National Institute for Medical Research, London, and issued as International Vitamin D Standard, should be retained. It also recommended that when the present International solution was exhausted, or if it should become unsatisfactory for any reason, it should be replaced by an equivalent solution of pure crystalline vitamin D in olive oil of such strength that 1 mg.

assay being used for the calculation of potency and of error of that assay.

Meanwhile, the need of a standard of reference for vitamin D was being recognised in other laboratories and in other countries. It was obviously desirable that workers in different laboratories should use the same standard and unit of activity. A second larger batch of irradiated ergosterol in olive oil had been prepared in 1928 at the National Institute for Medical Research under conditions similar to those of the first batch, part being kept at -4° to 0°C . and part at room temperature. A further large batch was prepared early in 1929 and extensive biological comparisons made between it and the second and first batches. It proved to have the same activity as the first batch and also the same as the part of the second batch which had been kept at the very low temperature. The part of the second batch which had been kept at room temperature, however, was very much less active than the third batch and a direct comparison between the two parts of the second batch confirmed the loss of activity of the part kept at room temperature. The Medical Research Council¹ therefore made available a standard of reference of vitamin D and recommended its adoption. It also recommended that the unit of activity should be defined as the antirachitic potency of a quantity of this preparation corresponding to 0.0001 mg. of the ergosterol used in its production. Thus the unit, as far as biological tests could determine, had the same value as the one already used for some years in the Pharmaceutical Society's laboratory. It was of convenient size for laboratory use on test animals; that is to say, the doses required for tests were neither a large multiple nor a small fraction of the unit proposed. The solution for distribution was made up to contain 1,000 units/g. It was ready for issue the following September, and a memorandum giving details for the use of the standard was sent out with the first issue to each laboratory proposing to use it. Thereafter, fresh supplies were sent out every 6 months. Still another large preparation of irradiated ergosterol was made in 1931 and by biological tests it was shown to be indistinguishable in antirachitic activity from the previous one.

The need for standards of reference for other vitamins also had become urgent, not only in Great Britain but also in other countries. In 1931 a conference on Vitamin Standards² was held in London at the invitation of the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations and standards of reference were adopted for International use for vitamins A, B₁, C and D.

The 1931 Conference recommended that the Standard solution of irradiated ergosterol at that time issued from the National Institute for Medical Research, London, should be adopted as International Vitamin D Standard for the next two years. If, within that period, it should become necessary, owing to exhaustion of the supply, to replace that solution by a fresh standard, the equivalence should be determined by experts of different countries. It also recommended for adoption as international

test will then be acceptable if it will give an accurate comparative measure of the quantity or proportion of that principle; and provided it so measures the right thing, and measures it accurately enough, the activity on which the measurement depends need have no more relation to the therapeutic action than would the property forming the basis of a chemical determination of the active principle, if such were applicable. The position becomes at once more complicated when several active principles are in question, which may be present in varying proportions, and concerning the relative therapeutic importance of which there is no sufficient ground for decision or differentiation...."

The Second Conference on the Standardisation of Vitamins held in 1934 recommended that "the experience gained in the use of cod-liver oils standardised against the International Standard by means of tests on rats might be devoted to an attempt to elucidate the questions involved in the anomalous action on certain species of different sources of vitamin D." It was Waddell¹⁰ who first threw light on this subject. Reverting to the antirachitic substance generated in cholesterol on irradiation, he found it to be at least as active as the vitamin D of cod-liver oil in the prevention of rickets in chicks, the respective doses being determined by tests on rats, and he concluded that the pro-vitamin D of cholesterol was not ergosterol. Work by Windaus, Lettre and Schenck, Grab, Brockmann, Brockmann and Busse led to the isolation of a substance from the products of the irradiation of 7-dehydro-cholesterol and its identification with the vitamin D of tunny-liver oil and halibut-liver oil. This substance was named vitamin D₃ and it was reported to have the same antirachitic potency (40,000 I.U./mg.) for rats as calciferol. A collaborative test to compare the antirachitic potencies of calciferol (vitamin D₂) and vitamin D₃ was organised by the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute.¹¹ Workers in 9 different laboratories in Great Britain made the comparison by means of tests on rats, and the relative potencies of the two preparations, as indicated by the different workers, were all so nearly equal to unity that statistical analysis of the result was considered unnecessary. Vitamin D₃ was declared to contain 40,000,000 International units of Vitamin D per gram. Moreover, a comparison of these two preparations on the healing of rickets in children carried out by Morris and Stephenson in Glasgow, on cases of osteomalacia and late rickets by Wilson in India and on a case of parathyroid tetany by Himsworth and Maizels in London failed to show any difference in antirachitic potency and, indeed, offered a certain amount of positive evidence that the two substances were equally potent for human beings. Various other workers had shown that the vitamin D of cod-liver oil (whatever might be its nature) and calciferol were equally potent for human beings. Thus it was possible to adopt vitamin D₃ as a standard of reference for determining the potency of oils intended for rats, chicks or human beings. The possibility of its adoption was to have been included in the Agenda of the Third International Conference

contained 0.025 μ g. of crystalline vitamin D, a definition for which was added. The unit recommended for adoption by the 1931 Conference was to remain unaltered: namely, the vitamin D activity of 1 mg. of the International Standard solution of irradiated ergosterol which had been found equal to that of 0.025 μ g. of crystalline vitamin D.

By this time it was becoming more and more evident that irradiated ergosterol had very little antirachitic activity for chicks; hence a determination of the vitamin D potency of a cod-liver oil by comparison with the standard by means of tests on chicks would give a very much larger value for the oil than a similar test carried out on rats. Therefore, the Conference stated that the assay of materials for vitamin D content should be carried out by comparative tests on rats, and their value in International units should be derived from the results of such tests. If other species were employed for these tests, the values could not be expressed in International units.

With regard to the method of determination, the 1931 Conference considered it permissible to use various biological methods of estimation, either prophylactic or therapeutic, e.g., the "line-test," X-ray examination or determination of the bone ash, provided that not less than (and preferably more than) 20 rats were used, one half receiving doses of the Standard and the other receiving doses of the substance under test. The Conference did not consider it desirable to draft detailed procedures for the determinations. Indeed, Dr. R. Gautier⁹, now Assistant Director-General to the World Health Organisation and in 1945 Secretary to the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations, wrote at that time (with regard to all biological assays), "When the value of an international unit has thus been given an agreed definition, it has been recognised that the nature and details of the biological method used in making the comparative measurements in units should be left, so far as possible, to the free choice and judgment of the expert conducting the test. Each worker is likely to make the most accurate assays when using a method with which experience and opportunity have made him familiar. Freedom of choice in this matter also affords an incentive to researches aiming at the improvement of existing methods and the discovery of better ones, whereas the adoption of a single method by agreement and the standardisation of its details must tend to stereotype knowledge and hinder progress in this field. There are certain conditions, however, by which the choice of a method of biological measurement must be guided. It should, of course, be capable of yielding results of the greatest practicable precision; but the fact is apt to be overlooked that the most accurately reproducible results are of doubtful value, and may even be misleading, unless it is certain that the method is measuring the therapeutically important constituent of the product under test. In a case where the assay can be assumed to be dealing with a single and uniform active principle, the position is simple. Any biological

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Britain, Denmark, Holland, Norway, Sweden, New Zealand, Canada and the U.S.A. did so. It was impossible to find experts able to carry out the comparisons in France, Spain, Portugal, India or South Africa. A most complete and exhaustive series of comparative tests was carried out and investigators in 33 laboratories sent in reports, some concerning tests with chicks, some with rats, some with both. Some even sent in duplicate tests with rats, one to compare results obtained with albino and piebald rats respectively.

The British participants and Dr. J. O. Irwin met to arrange the design of the experiment, and a scheme put forward by N. T. Gridgeman was adopted. It entailed randomisation of litters between doses (x, 2x, 4x) and randomisation of rats within each litter between the four samples. Thus all rats of any one litter had the same size of dose, but of different preparations. Altogether there were to be at least 10 litters on each dose, i.e., at least 30 litters in all, comprising at least 120 rats. Details of procedure (diet, criterion, duration of test, etc.) were left to individual workers. The work in the U.S.A. and Canada was organised by the U.S.P. Committee, who accepted the design planned by the British workers and issued certain instructions to their participants to accord with their own experience. In addition, they issued 2 bottles of cod-liver oil, 1 to be assayed on the assumption that it contained 115 U.S.P. units (International units)/g. and the other as 96 U.S.P. units (International units)/g. Actually these were one and the same oil, but it seemed a good opportunity for investigating the possible influence of "level" of testing on the result obtained.

In all, 54 assays were completed, 29 using rats and 25 using chicks. Dr. J. O. Irwin and his colleagues calculated the results of each test and its fiducial limits of accuracy and the ratios of the potencies (with their fiducial limits) of the various preparations. Their summary¹³ is as follows:—

	Result	Fiducial Limits (P = .95)
(a) New Standard/Old Standard	0.981	0.925 to 1.009
(b) B.S.I. Standard/Old Standard	0.916	0.902 to 0.983
(c) Calciferol/Old Standard	0.933	0.896 to 0.972
(d) U.S.P. Ref. C.L.O./Old Standard...	0.869	0.839 to 1.004
(e) New Standard/B.S.I. Standard	1.071	1.036 to 1.098
(f) U.S.P. Ref. C.L.O./B.S.I. Standard	0.949	0.843 to 0.958

Irwin says, "Thus the B.S.I. Standard and the calciferol are less potent than the Old Standard and the New Standard may be slightly so. The new standard is somewhat more potent than the B.S.I. Standard, while the calciferol and B.S.I. Standard do not differ significantly. The U.S.P. Reference Oil is less potent than the B.S.I. Standard. This could not be concluded from the rat assays only, and so may be due to the presence of a little vitamin D₂ in the oil. The results are remarkably uniform. For (a), (b), (c), the fiducial range is less than 10 per cent., for (e) it is cent., for (d) and (f) it is rather greater, but still of a r. for a biological test."

on Vitamin Standardisation planned for September, 1939, but not held at that time on account of the outbreak of war.

Meanwhile the British Standards Institution had adopted a particular sample of vitamin D₃ as a Provisional Standard Preparation for assaying the vitamin D potency of cod-liver oils intended for chick feeding. It was dissolved in pure vitamin D-free olive oil; 1 mg. of the solution contained 0.000025 mg. of the crystalline vitamin, and this amount was adopted as the B.S.I. unit of antirachitic activity. Since rats appear to use vitamin D₂ and vitamin D₃ equally well, 1 unit of the B.S.I. Standard was equivalent to 1 International unit as far as rats were concerned. It was obvious, however, that a cod-liver oil intended for chick feeding must be assayed by tests on chicks, for a cod-liver oil containing added calciferol would have a high value according to tests on rats, but would be less potent when given to chicks. The British Standards Institution drew up a plan¹² for determining the vitamin D content of cod-liver oils by using chicks as test animals and the ash content of the bone as criterion. An example worked out for potency and approximate and fiducial errors was added. The result of such a test, however, could only be expressed in B.S.I. units since the oil was compared with the B.S.I. Standard.

The work carried out on this B.S.I. Standard in many laboratories in Britain, Canada and the U.S.A. proved of the greatest value and paved the way towards the adoption of an International Standard of vitamin D₃. As soon as the World Health Organisation was formed, the International Standards for vitamin A and vitamin D came under review. The story of the vitamin A standard will be told later. Work for this vitamin D standard began in 1946. The Accessory Food Factors Committee of the Medical Research Council and Lister Institute organised, through its vitamin D sub-committee, a large collaborative experiment to investigate the possibility of adopting vitamin D₃ as the International Standard of reference for vitamin D. Several manufacturers were making crystalline vitamin D₃, and samples of this substance were generously contributed by them. The total weight contributed was about 29 g. The physical constants of each sample were determined. The samples were pooled, and the physical constants of the pooled sample were determined. The solution for issue was prepared at the National Institute for Medical Research, London, and was of the same strength as that issued by the B.S.I., viz., 0.000025 mg. in 1 mg. of oil. It was decided at a meeting of the Vitamin D sub-committee to compare the following preparations:—

1. The present International Standard for vitamin D (irradiated ergosterol in olive oil).
2. The new preparation of pooled samples of vitamin D₃.
3. The British Standards Institution Standard for vitamin D₃.
4. A preparation of the purest sample of calciferol obtainable.

The Committee invited laboratories in as many countries as possible to take part in the comparative tests. Eventually workers in Great

THE INTERNATIONAL STANDARD FOR VITAMIN D

Britain, Denmark, Holland, Norway, Sweden, New Zealand, Canada and the U.S.A. did so. It was impossible to find experts able to carry out the comparisons in France, Spain, Portugal, India or South Africa. A most complete and exhaustive series of comparative tests was carried out and investigators in 33 laboratories sent in reports, some concerning tests with chicks, some with rats, some with both. Some even sent in duplicate tests with rats, one to compare results obtained with albino and piebald rats respectively.

The British participants and Dr. J. O. Irwin met to arrange the design of the experiment, and a scheme put forward by N. T. Gridgeman was adopted. It entailed randomisation of litters between doses (x, 2x, 4x) and randomisation of rats within each litter between the four samples. Thus all rats of any one litter had the same size of dose, but of different preparations. Altogether there were to be at least 10 litters on each dose, i.e., at least 30 litters in all, comprising at least 120 rats. Details of procedure (diet, criterion, duration of test, etc.) were left to individual workers. The work in the U.S.A. and Canada was organised by the U.S.P. Committee, who accepted the design planned by the British workers and issued certain instructions to their participants to accord with their own experience. In addition, they issued 2 bottles of cod-liver oil, 1 to be assayed on the assumption that it contained 115 U.S.P. units (International units)/g. and the other as 96 U.S.P. units (International units)/g. Actually these were one and the same oil, but it seemed a good opportunity for investigating the possible influence of "level" of testing on the result obtained.

In all, 54 assays were completed, 29 using rats and 25 using chicks. Dr. J. O. Irwin and his colleagues calculated the results of each test and its fiducial limits of accuracy and the ratios of the potencies (with their fiducial limits) of the various preparations. Their summary¹³ is as follows:—

	Result	Fiducial Limits (P = 95)
(a) New Standard/Old Standard .	0.981	0.925 to 1.009
(b) B.S.I. Standard/Old Standard	0.916	0.902 to 0.983
(c) Calciferol/Old Standard . . .	0.933	0.896 to 0.972
(d) U.S.P. Ref. C.L.O./Old Standard...	0.869	0.839 to 1.004
(e) New Standard/B.S.I. Standard	1.071	1.036 to 1.098
(f) U.S.P. Ref. C.L.O./B.S.I. Standard	0.949	0.843 to 0.958

Irwin says, "Thus the B.S.I. Standard and the calciferol are less potent than the Old Standard and the New Standard may be slightly so. The new standard is somewhat more potent than the B.S.I. Standard, while the calciferol and B.S.I. Standard do not differ significantly. The U.S.P. Reference Oil is less potent than the B.S.I. Standard. This could not be concluded from the rat assays only, and so may be due to the presence of a little vitamin D₂ in the oil. The results are remarkably uniform. For (a), (b), (c), the fiducial range is less than 10 per cent.; for (e) it is about 6 per cent., for (d) and (f) it is rather greater, but still of a satisfactory order, for a biological test."

A Sub-Committee on Fat-soluble Vitamins of the Expert Committee on Biological Standardisation of the World Health Organisation held a meeting in London in 1949¹³, and recommended that the preparation of crystalline vitamin D₃, as described below, at present held at the National Institute for Medical Research, London, should be adopted as the International Standard for vitamin D. This new standard should replace the existing solution of irradiated ergosterol and the latter should be retained as a reference preparation only and not as an International Standard. The Committee also recommended that the International Unit of vitamin D should be the vitamin D activity of 0.025 µg. of the International Standard preparation of crystalline vitamin D₃. The Standard should be issued as a solution containing 1000 I.U. per gram. The properties of the recrystallised vitamin D₃ freshly withdrawn from a sealed ampoule were determined as:—

m.pt. 87° to 89°C. (corr.)

$[\alpha]_D^{20^\circ C} = +110^\circ$ (ethanol)

$E_{1\%}^{1\text{cm}} = 265 \text{ m}\mu = 490$ (ethanol) corresponding to a molecular extinction coefficient of 18,800.

The recommendations of the sub-committee were adopted by the General Assembly of the World Health Organisation held in Rome in June, 1949.

Step by step with the growing use of international standards of reference for assaying the biological potency of therapeutic substances has been the adaptation of existing methods and development of further methods and refinements of methods for estimating the accuracy of assays. The first edition of Fisher's "Statistical Methods for Research Workers" was published in 1925. It has now reached its tenth edition; several other writers have published somewhat similar books and a few mathematicians, such as J. O. Irwin, E. C. Fieller, D. J. Finney and C. I. Bliss, have applied themselves to original investigations in this subject. Biologists have followed the trail these pioneers have blazed and now they know just how much as well as how little faith they may place in their results. The one is quite as important as the other.

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BRITISH PHARMACEUTICAL CONFERENCE BLACKPOOL, 1949

RESEARCH PAPERS

PENICILLIN FORMULATIONS: THE EFFICACY OF OILY INJECTIONS

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THE rapid excretion of penicillin when administered parenterally in aqueous solution has led to the development of several non-aqueous formulations designed to prolong the therapeutic blood level after injection. The object of "slow-release" is two-fold; firstly to eliminate the inconvenience and pain to the patient of frequent injections and secondly to avoid wastage of penicillin. Its adoption is based on the now generally accepted principle that a continuous therapeutic level is more effective than intermittent "peaks." The three main principles so far employed in such preparations are: (a) the suspending of penicillin in vegetable oil, usually in presence of a thickening or dispersing agent, (b) the utilisation in these suspensions of penicillin salts which are only sparingly soluble in water, and (c) the control of particle size of the suspended penicillin. The first slow-release formulation was devised by Romansky¹, who suspended a water-soluble salt of penicillin in arachis oil containing 4.8 per cent. of beeswax. Numerous clinical reports have shown that this preparation achieves therapeutic levels (>0.03 u./ml.) for periods up to 24 hours after injection. Later, the preparation of sparingly soluble salts of penicillin containing either heavy-metal or organic bases was followed by their trial in slow-release formulations. Of these, the most effective so far has been procaine penicillin. Clinical reports on preparations containing procaine penicillin suspended in untreated arachis oil were made by Herrell, Nichols and Heilmann², and by Hobby Brown and Patelski³, both claiming therapeutic levels for periods greater than 24 hours. At about the same time, attention was drawn to the significance of the particle size of the suspended penicillin by Romansky and Dowling⁴, who reported that in oil/wax suspensions of soluble salts the best results were obtained when the particle size of the latter exceeded 50μ . This view was supported by Sullivan⁵, who used large particle size procaine penicillin in untreated arachis oil. Shortly after the introduction of procaine penicillin Buckwalter and Dickison⁶ developed a new technique of suspending penicillin by using an aluminium stearate/arachis oil gel instead of oil/wax or untreated oil. Clinical reports on the new suspensions were made by Thomas and his co-

workers⁷, who obtained therapeutic levels for as long as 96 hours but who simultaneously showed that small particle size penicillin was more effective than large in stearate/oil suspensions. Beyond relatively simple theories such as "water-proofing" of penicillin, no satisfactory explanation of the mechanism of slow-release has been advanced. A survey of earlier work also shows that not all the variants of the slow release formulations have a common basis for comparison. This is largely due to the fact that clinical and laboratory evaluation has been by a variety of techniques. The object of the work described here was to compare as accurately as possible the efficacy of a series of preparations embodying the most important of the possible variants, viz. (a) soluble and sparingly soluble penicillin (potassium and procaine salts), (b) gelling and suspending agents (beeswax and aluminium stearate), and (c) particle size of penicillin. To permit comparison of a large number of variants with sufficient accuracy it was decided to adopt a laboratory animal test for evaluation, and to use statistical methods for the efficient design of the experiments and analysis of the results.

EXPERIMENTAL

This consisted essentially of two groups of tests described here as Experiments 1 and 2. In both experiments the orders of preparation of samples, dosing and inoculation were randomised to ensure that any extraneous non-random variation would not vitiate the comparison between the main factors of the experiments. The following standard techniques were used:—

(1) *Biological Evaluation.* Three groups, each of 10 mice, were injected subcutaneously with the test preparation, each mouse receiving 30,000 units in 0.1 ml. The mice were then infected intraperitoneally with a 24-hour culture of *Streptococcus pyogenes* Krüger strain. All three groups were infected simultaneously, but the test preparation was injected in advance at different time intervals for the three groups. The usual plan was to inject the first group 5 days before infection, the second 3 days, and the third 2 days. The number of deaths occurring up to 72 hours after infection was recorded for each group. A similar test has been used by Miller⁸. In general the number of deaths increased with the time interval between dosage and infection and the criterion used for comparing the effectiveness of the different formulations studied here was the estimated time between dosage and subsequent infection required to produce a 50 per cent. mortality in the mice. The greater this time the more effective the preparation.

(2) *Determination of Penicillin Potency of Test Preparation.* The penicillin salts were assayed both microbiologically (*Staphylococcus aureus*) and iodimetrically⁹. Suspensions were assayed iodimetrically only and the results interpreted by means of a factor obtained in the assay of the penicillin salt.

(3) *Control and Measurement of Particle Size of Penicillin.* Three ranges of particle size were selected for testing and are described here

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arbitrarily as "coarse," "medium," and "fine." Reduction to appropriate particle size was effected by dry grinding, followed, where necessary, by grinding in oil (with subsequent removal of oil before incorporation in the arachis oil base). Representative particle size analyses are expressed graphically in Figure 1, the measurements being made using a technique based on that of Fairs¹⁰. The graph expresses the relationship between the ranges and shows that the distribution in each range does not exhibit any unusual feature.

(4) *Preparation of Base.* (i) *Stearate/Arachis Oil:* Four methods were used, differing essentially in the temperatures at which the oil and stearate

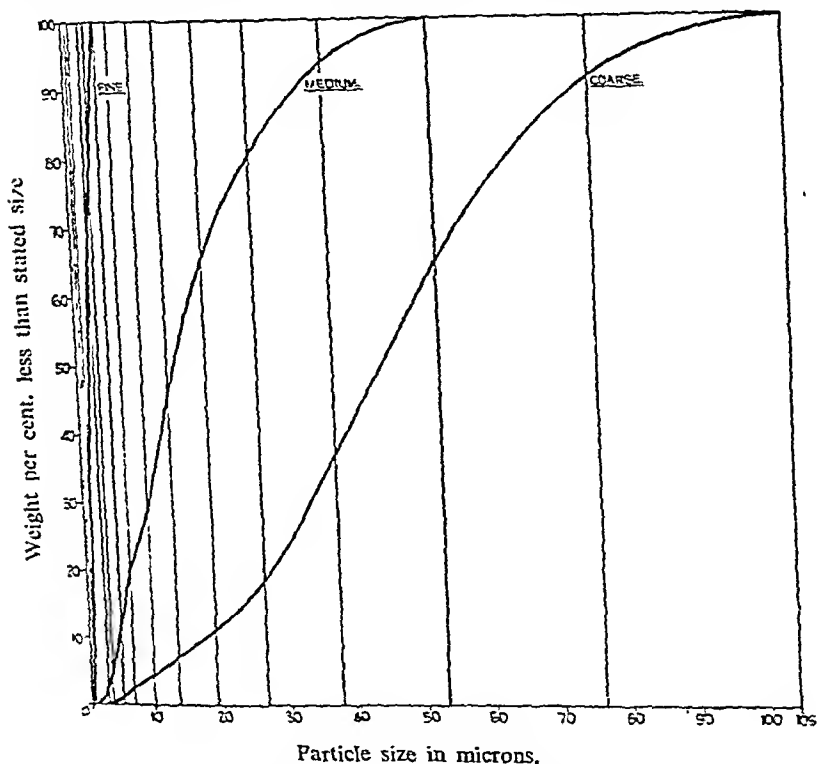


FIG. 1. Particle size distribution of typical preparations.

were gelled. (a) *High temperature:* Powdered aluminium stearate was dispersed in arachis oil, and the mixture heated slowly and with constant stirring to within the range 117° to 122°C. At this point the mixture became extremely viscous. The temperature was then raised to 150°C. and maintained at this level for 1 hour to effect sterilisation. The final product was rather less viscous than at 117°C. (b) *Medium temperature:* Aluminium stearate and arachis oil were separately sterilised by maintaining at 140°C. for 4 hours. The stearate was then dispersed in the oil, using aseptic technique, and the mixture raised slowly, and with stirring, to within the range 125° to 130°C. At this temperature the mixture

gelled and developed maximum viscosity, which was retained on cooling. (c) *Low temperature*: As for method (b), but the gelling temperature was raised to within the range 117° to 122°C. This gave a gel of slightly higher viscosity than (b). (d) *Unheated*: Aluminium stearate and arachis oil were separately sterilised by maintaining at 140°C. for 4 hours and then mixed by ball-milling for 24 hours. This yielded a fine dispersion of stearate in oil approximately equal in viscosity to the original oil.

(ii) *Beeswax/Arachis Oil*: The beeswax and arachis oil were mixed in the cold and the temperature raised slowly, with stirring, to 150°C., at which temperature it was maintained for 1 hour. The solution was allowed to cool and was agitated gently during cooling from 60°C. to room temperature to ensure dispersion of beeswax in a finely divided state.

(5) *Incorporation of Penicillin in Beeswax or Stearate Base*: The penicillin was mixed with the prepared sterile base in a mortar and the mixture transferred to a standard vessel containing a fixed charge of steel balls. The vessel was sealed and rotated for 1 hour, the speed being adjusted so as to ensure efficient mixing with minimum alteration of particle size.

Experiment No. 1: Using the standard techniques already described, the effect of varying the following factors was investigated: (a) penicillin salt (procaine and potassium), (b) nature of suspending agent (beeswax and aluminium mono-, di-, and tristearates), (c) concentration of suspending agent (1 per cent. and 2 per cent. for both beeswax and stearate), (d) particle size of penicillin (medium and fine). The samples of aluminium stearate used are described here arbitrarily as mono-, di-, and tri-stearates since analysis showed that the base/acid ratio was approximately of this order. Typical analyses are given in the Appendix. All stearate/oil gels were prepared by the high temperature method, variations in the method of gelling being studied in Experiment 2. It was not considered desirable at this stage to examine the four factors in all combinations, and in the first part of the experiment (1a) one half of the combinations representing a half replicate of the full factorial design¹¹ was

TABLE I

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT MORTALITY

Penicillin Salt	Particle Size	Monostearate	Distearate	Tristearate	Beeswax
		0 36 percent (1)	0 85 percent (2)	0 55 percent (2)	-0 14 percent (1)
Procaine ..	Medium			0 99 (1)	0 57 (2)
	Fine	0 68 (2)	0 44 (1)		
Potassium ..	Medium	0 14 (2)	-0 48 (1)	All mice died (1)	All mice died (2)
	Fine	0 01 (1)	0 14 (2)	0 16 (2)	All mice died (1)

The figures between the brackets refer to the concentration of stearate or beeswax

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carried out. The results are given in Table I expressed as logarithms of the time required between dosage and subsequent infection to produce 50 per cent. mortality. In deriving these results it was found that the mortality measured as a probit was linear with respect to the logarithm of the time between dosage and infection. The well-known method of probits¹² was then used to calculate the 50 per cent. point. The slope of the probit log-time line was calculated for each sample. Each slope had a fairly large standard error and the assumption could be made that all the slopes were equal. The combined slope was then calculated and used to obtain an estimate of the 50 per cent. point for all preparations.

The reliability of the results differed from sample to sample but the variation was not large and an average figure of 0.14 for the standard deviation could be used without risk of serious error. It is clear from this table that potassium is much inferior to procaine, and beeswax inferior to aluminium stearate but the 6 remaining samples of procaine penicillin in stearate gels do not give sufficient information on the comparison between the particle size of penicillin and the three types of stearate. A further 6 samples were therefore prepared (Experiment Ib), with the 2 different particle sizes of procaine penicillin, using the three stearates at concentrations of 1 per cent. and 2 per cent. but with these concentrations reversed. Table II shows the results of this experiment together with the relevant portion from Table I. The two halves of the experiment then consisted of samples prepared using all combinations of the variables, particle size, concentration, and type of stearate.

TABLE II

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT. MORTALITY

	Particle Size	Monostearate		Distearate		Tristearate	
			percent.		percent.		percent.
Experiment Ia	Medium	0.36	(1)	0.85	(2)	0.55	(2)
	Fine	0.68	(2)	0.44	(1)	0.99	(1)
Experiment Ib	Medium	0.32	(2)	0.32	(1)	0.34	(1)
	Fine	0.28	(1)	0.11	(2)	0.18	(2)

The figures between the brackets refer to the concentration of stearate.

It should be noted that the average result for Experiment Ib is appreciably lower than that for Experiment Ia. This is probably due to the different batch of mice or the different suspension of infecting organism, or both, the experiments being carried out at different times. The design is such, however, that all the important comparisons are made within experiments and the comparison between the experiments has been made to coincide with the higher and less important interactions between the factors. The analysis of variance is given in Table II.

The standard deviation of each result calculated from the detailed observation on the mice is approximately the same for both experiments, the average value being 0.130, which corresponds to a variance of 0.0169.

From Table III it is seen that the efficacy of the preparations is influenced by the amount of the stearate used. The magnitude of this

effect is shown by a comparison of the averages for all the 1 per cent and all the 2 per cent. preparations given in Table II: mean for 2 per cent. stearate, 0.549; mean for 1 per cent. stearate, 0.355. The 2 per cent preparations are clearly more effective.

Table III also shows that efficacy is influenced by the particular combination of type of stearate and particle size of the procaine penicillin

TABLE III
ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	D/F	Variance
Type of stearate	0.0258	2	0.0129
Amount of stearate	0.1121	1	0.1121
Particle size	0.0003	1	0.0003
Interactions —			
Stearates with particle size	0.1347	2	0.0674
Stearates with amount	0.0230	2	0.0115
Between experiments	0.4454	1	0.4454
Remainder	0.0440	2	0.0220
Total	0.7853	11	—
Error	—	Large	0.0169

used. This is seen more clearly in Table IV, in which the results of Experiments 1a and 1b have been averaged.

Table IV shows that fine particles are better with mono- and distearate, but worse with distearate, and that medium particles are best with distearate. This type of interaction was unexpected and requires verification. Should this interaction be fortuitous, it is unlikely to affect the further conclusion that fine particles are not significantly different from medium particles. Table IV also shows an apparent trend to higher results with increasing stearic acid content of the aluminium stearate

TABLE IV
(a) INTERACTION OF TYPE OF STEARATE WITH PARTICLE SIZE

Particle Size	Type of Stearate			Means
	Mono	Di	Tri	
Medium	0.340	0.584	0.445	0.456
Fine	0.478	0.275	0.588	0.447
Mean	0.410	0.430	0.516	

Experiment No. 2: This was designed to assess the effect of varying (a) the gelling method (high, medium and low temperature and unheated), (b) the type of aluminium stearate (aluminium stearate of no specified composition and an aluminium stearate of no specified composition referred to as "Technical"), (c) concentration of stearate (2 per cent, referred to as "Technical"), (d) particle size of procaine penicillin (medium and 3 per cent) and (e) statistical design used to examine these factors was a 2⁵ factorial design. This involved 16 samples, and the results expressed as logarithms of the estimated time between dosage and results expressed as mortality in mice are given in subsequent table V.

PENICILLIN FORMULATIONS: THE EFFICACY OF OILY INJECTIONS

TABLE V

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT. MORTALITY

Particle Size	Type of Stearate	High Temperature	Medium Temperature	Low Temperature	Unheated
		percent.	percent.	percent.	percent.
Coarse ...	Mono-Technical ...	0.40 (3)	0.25 (2)	0.39 (3)	-0.23 (2)
		0.49 (2)	0.38 (3)	0.40 (2)	-0.35 (3)
Medium ...	Mono-Technical ...	0.50 (2)	0.43 (3)	0.60 (2)	0.06 (3)
		0.56 (3)	0.44 (2)	0.44 (3)	-0.03 (2)

The analysis of variance of these results is given in Table VI.

The only significant effects are particle size of penicillin and temperature of gelling. The residual variance, which is a mixture of interactions, is no greater than the error variance due to the inherent variability of the mice. Had the reverse been the case, it would have been necessary to carry out the second half replicate to complete the factorial design

TABLE VI
ANALYSIS OF VARIANCE

Source of Variation							Sum of Squares	D/F	Variance
Concentration of stearate	0.0038	1	0.0038
Particle size	0.0848	1	0.0848
Type of stearate	0.0002	1	0.0002
Temperature of gelling	1.1367	3	0.3789
Residual	0.0460	9	0.0051
Total	1.2715	15	—
Error	—	Large	0.0177

involving all combinations of the factors. The mean results are as follows:—

Particle Size: Coarse = 0.217; Medium = 0.362.

Temperature of gelling: High = 0.489; Medium = 0.378; Low = 0.485; Unheated = - 0.167.

Medium particle size is thus better than coarse, and unheated gels inferior to heated. There is no clear distinction between the high, medium and low temperatures of gelling. Similarly, there is no detectable difference between the two types of stearate and between 2 per cent. and 3 per cent. concentrations of stearate.

DISCUSSION

The reliability of the mouse protection test has already been discussed by Miller⁸, whose conclusions are confirmed by comparison of his results and those reported here with the clinical data of Thomas⁷. Assuming that the test gives a good indication of the prolongation of blood levels achievable in man, it would thus appear that the most efficient of the oily injections so far examined consists of procaine penicillin (fine particle size) dispersed in oil gelled by aluminium stearate. Whether the three factors of penicillin salt, particle size, and gelling agent are closely inter-

related is not yet established, but the superiority of procaine penicillin over the potassium salt clearly extends to all formulations and all particle sizes. As far as penicillin salt is concerned, this might lead to a theory of delayed absorption due to reduced water-solubility. This explanation cannot be accepted, however, until a series of water insoluble salts have been studied. It has already been shown⁷ that aluminium penicillin is less effective than the procaine salt and this difference cannot be explained on the grounds of solubility alone. The influence of particle size presents an interesting problem. The first reports^{4,5} favoured large particles but the position was reversed when stearate gels were introduced, suggesting that the action of the latter is connected with the surface area of penicillin. The fact that gels of different viscosity do not differ significantly in biological behaviour indicates that aluminium stearate does not merely act as a thickening agent, although it is equally clear that it loses its effect if gelling does not take place. The chemical composition of the stearate is apparently not a significant factor and therefore samples may be selected on purely pharmaceutical grounds without risk of sacrificing activity. The optimum proportion of stearate seems to be approximately 2 per cent. of the oil base. Preparations containing more than this have no increased activity and are more difficult to manipulate.

In the light of the foregoing, it is suggested that the mechanism of aluminium stearate probably involves the formation of a protective film around the penicillin particles. Such a film might be created by interaction at the penicillin surface to produce "procaine stearate," the molecules of which could be orientated with the carbon chain in the oil phase. This would retain a film of oil over the penicillin surface, the former being held more tenaciously than would be a film of untreated oil. This theory would explain the effect of penicillin particle size since the area of adsorbed film would increase with reduction of the particle size. The theory is also compatible with the other observed effects of concentration of stearate and disappearance of activity in non-gelled material. In the former case the optimum concentration required to establish the protective film is apparently of the order of 2 per cent., and nothing is gained by raising this figure. For the latter, it is suggested that the interaction with procaine penicillin can only take place when the stearate is in the gelled state.

SUMMARY

(a) A comparison has been made of the efficiency of various oily injections of penicillin using a mouse-protection test.

(b) It is concluded that the most satisfactory preparation consists of procaine penicillin of small particle size dispersed in a stearate/arachis oil gel.

(c) A theory is suggested for the mechanism by which aluminium stearate induces "slow-release."

I am indebted to Dr. O. L. Davies for the design of the tests and the analysis of results, to Dr. A. R. Martin for supervision of the biological work, and to Mr. A. G. Fishburn for help in the preparation of the paper.

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APPENDIX

ANALYSIS OF ALUMINIUM STEARATES

Test	Monostearate	Distearate	Tristearate	Technical
	per cent.	per cent.	per cent.	per cent.
Loss at 100°C. for 3 hours ...	1.5	0.66	0.96	0.9
Ash ..	16.20	8.75	6.20	0.05
Water-insoluble ash ..	15.90	8.2	5.3	9.69
Aluminium (as Al ₂ O ₃) ..	16.2	8.8	6.4	10.10

DISCUSSION

The CHAIRMAN said that the paper was an interesting illustration of the use of the mouse protection test as an alternative to the rabbit method of studying the persistence of penicillin in the blood. It was also a very good example of the value of physical chemistry in pharmacy.

DR. K. BULLOCK (Manchester) observed that procaine penicillin had been chosen from a number of salts of penicillin and organic bases. Could the author give the references to the other organic bases or indicate the types of other organic bases tried?

PROFESSOR H. BRINDLE (Manchester) said he was interested in slow release vehicles for penicillin and in the use of the mouse protection test as a criterion. He had always used the rabbit and, in common with other workers, had found some difficulty in getting a sample of the blood which was sufficiently sterile to give satisfactory results.

In the mouse protection test the solution or suspension was injected subcutaneously into the mouse. Everything depended on the relationship between the oily depot and the surrounding tissue and this might give very different results from those which one would get by intramuscular injection in human beings. He had not consulted the references cited by the author but thought that Thomas and Miller had worked with aqueous solutions, which were very different from oily suspensions. Did the author think it quite fair to relate his results on mice to what might happen when suspensions were administered intramuscularly in human beings?

DR. R. E. STUCKEY (London) said that various papers had been published recently giving varying opinions on the efficacy of penicillin suspensions. Had the author any experience with the aqueous procaine penicillin suspension tested by his method?

DR. F. HARTLEY (London) mentioned that the author suggested that the viscosity of the solution made very little difference to the duration of action of the material. Did the extent of gelling vary with the age of the suspension? If so, the ease of manipulation would also vary.

MR. J. C. FLOYD, in reply, said that a number of salts were mentioned in the literature, and they had used a number of organic bases, including *p*-chlorophenyl biguanide. In reply to Professor Brindle, he thought that the question was really concerned with whether the test gave results comparable with those obtained in man. He thought this question was answered by Thomas's findings which showed excellent correlation between the results in mice and in human beings.

They had examined the aqueous procaine penicillin suspension by the method described, but the results were not very good; the effect was so rapid that the test became too insensitive.

He was not quite clear what Dr. Hartley meant by the extent of gelling. If one gelled under optimum conditions that gel on standing would eventually break. He thought that all aluminium stearate gels eventually broke on standing, and presumably to incorporate penicillin into a broken gel would be a much more simple matter than to incorporate it into an extremely viscous one. Whether there was any biological difference between a gel before and after breaking he could not say.

THE EFFECT OF THE SULPHONE GROUP IN HYPNOTICS

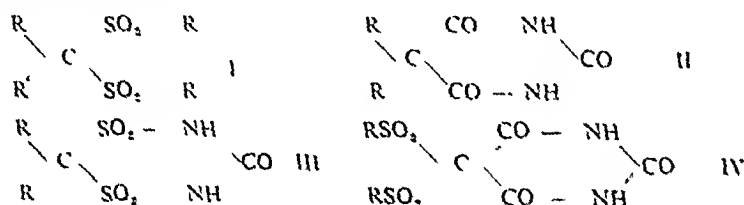
BY M. A. GHAMRAWI AND F. SAID

From the Chemistry Research Laboratories, School of Pharmacy,
Faculty of Medicine Fouad Ist University, Cairo, Egypt.

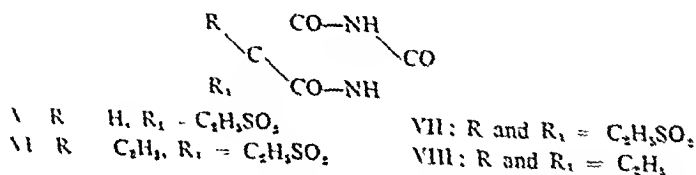
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THE EFFECT OF THE SULPHONE GROUP IN HYPNOTICS

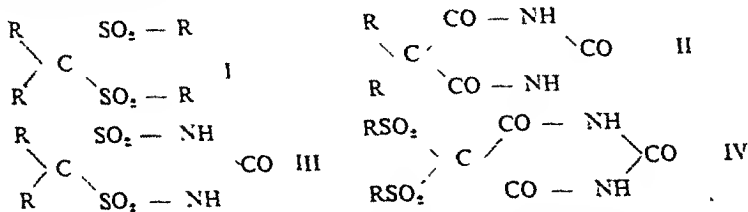
By M. A. GHAMRAWI AND F. SAID

*From the Chemistry Research Laboratories, School of Pharmacy,
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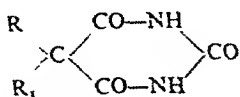
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V R H, R₁ = C₂H₅SO₂

VI: R = C₂H₅, R₁ = C₂H₅SO₂

VII: R and R₁ = C₂H₅SO₂

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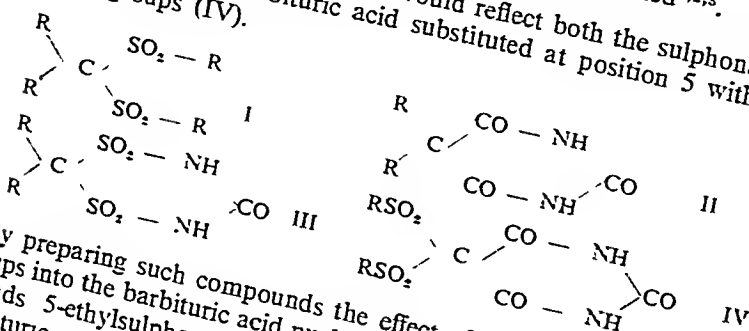
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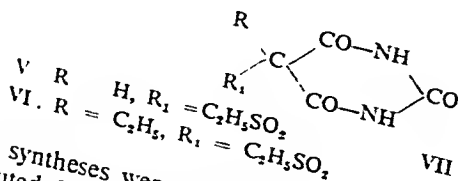
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VII: R and $\text{R}_1 = \text{C}_2\text{H}_5\text{SO}_2$
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We are indebted to Professor M. Sherif of the Pharmacology Department of the Faculty for the biological investigation of the new barbiturates. The relative activities of the compounds are tabulated as follows taking the activity of veronal as a reference.

Evidently, therefore, the introduction of sulphone groups into the molecule of barbitone has decreased its activity. The toxicity of the new products is greater than that of barbitone, thus the possibility of their administration may be restricted and unsafe.

EXPERIMENTAL

Sodium ethanesulphonate was prepared by a modification of the method of Hemillian⁵. A clear solution of anhydrous sodium sulphite (35 g.) in water (250 ml.) and ethyl iodide (30 g.) were heated on a water-bath under a reflux condenser until all the iodide disappeared (7 hours).

COMPOUND	SO ₂ GROUPS	ACTIVITY
$ \begin{array}{c} \text{Et} \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \quad \text{CO} \\ \diagup \quad \diagdown \\ \text{Et} \quad \text{CO-NH} \end{array} $	0	100
$ \begin{array}{c} \text{Et} \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \quad \text{CO} \\ \diagup \quad \diagdown \\ \text{EtSO}_2 \quad \text{CO-NH} \end{array} $	1	80
$ \begin{array}{c} \text{EtSO}_2 \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \quad \text{CO} \\ \diagup \quad \diagdown \\ \text{EtSO}_2 \quad \text{CO-NH} \end{array} $	2	10

Copper sulphate solution (100 ml. of 20 per cent.) was added with stirring to the warm reaction mixture and the precipitate so formed removed by filtration. The filtrate was neutralised with sodium hydroxide solution and concentrated on a water-bath till a scum formed on the surface, then allowed to crystallise, and filtered again. The filtrate was evaporated to dryness, the residue extracted with boiling alcohol (80 per cent.) and filtered whilst hot. On cooling the alcoholic filtrate sodium ethanesulphonate crystallised in thin wide plates. Yield, 78 per cent.

Ethylsulphonyl chloride was prepared by a modification of the method of Gerhardt⁶. Sodium ethanesulphonate (13 g.), dried at 120°C. under reduced pressure for 2 hours, was finely powdered and mixed with phosphorus pentachloride (20 g.) and then heated on a water-bath for 10 minutes. The cooled mixture was decomposed by pouring into ice-cold water and the acid chloride produced extracted with ether. After drying the extract and removal of the solvent, the acid chloride was distilled at 75° to 77°C./25 mm.Hg. Yield, 68 per cent.

EFFECT OF THE SULPHONE GROUP IN HYPNOTICS

Ethylsulphonylmalonic Ester (I). Diethylmalonate (30 g.) was added gradually with stirring to sodium wire (2.3 g.) covered with ether (150 ml.), the whole being heated on a water-bath under a reflux condenser. Slow addition of ethanesulphonyl chloride (10 g.) dissolved in ether (50 ml.) commenced after all the sodium had disappeared (1½ hours), stirring and refluxing being continued during the addition and for a further 7 hours until the mixture became neutral to litmus. Water was then added, the mixture acidified, the ethereal layer separated and the aqueous layer repeatedly washed with ether. The mixed ethereal solutions were dried over calcium chloride, the ether removed, and the residue distilled *in vacuo*.

Ethyl-ethylsulphonylmalonic ester (II) was prepared by the same procedure as I using ethylsulphonylmalonic ester (30 g.) in place of malonic ester and ethyl iodide (20 g.) in place of ethylsulphonyl chloride.

Diethylsulphonyl-malonic ester (III) was prepared by the same procedure as I using ethylsulphonylmalonic ester (30 g.) in place of the malonic ester.

All the esters occurred in the form of pale yellow oils soluble in ether and organic solvents, insoluble in water and showing a sp. gr. greater than 1. Table I gives their constants.

TABLE I

Ester	Boiling point	Sp. Gr at 20°C	Sulphur found	Content required	Yield
					per cent.
I	145 to 147 C /5 mm Hg	1.36	12.68	12.7	48
II	160 to 163 C /5 mm Hg	1.42	11.40	11.43	42
III	172 to 174 C /5 mm Hg	1.48	18.41	18.6	29

Condensation with Urea.

5-Ethylsulphonylbarbituric Acid (IV). Sodium (5.1 g.) was dissolved in absolute methyl alcohol (120 ml.). To the solution of 1 ml. of ethyl acetate was added followed, after 10 minutes at 60°C., by urea (12.7 g.). When the urea had dissolved, ethylsulphonylmalonic ester (15 g.) was added and the apparatus transferred to an oil-bath. A condenser for distillation and a sealed stirrer were fitted. The temperature of the reaction mixture was gradually raised to 130°C., in the course of 3½ hours, then the viscous mass was treated with ice and ice-cold water; when the bulk of the melt had dissolved, the alkaline solution was treated with 20 ml of benzene, then rendered distinctly alkaline to congo red with hydrochloric acid, concentrated and allowed to crystallise. The crude barbituric acid was recrystallised from an acetone-benzene mixture.

5:5-Diethylsulphonyl-barbituric acid (V). The above procedure was

applied using diethylsulphonemalonic ester (21 g.) in place of the monoethylsulphonemalonic ester. The product was recrystallised from 95 per cent. alcohol.

5-Ethyl-5-ethylsulphonylbarbituric acid (VI). (a) The same procedure was applied using ethyl-ethylsulphonylmalonic ester; the crude product was crystallised from 85 per cent. alcohol. (b) 5-Bromo-5-ethylbarbituric acid⁷ (2.7 g.) and sodium ethanesulphinate⁸ (1.2 g.) were dissolved in absolute methyl alcohol (20 ml.) and the mixture refluxed for 8 hours. The precipitated sodium chloride was filtered and the filtrate concentrated and allowed to crystallise. The product was identical with that above as confirmed by a mixed melting-point.

All the barbituric acid derivatives obtained were crystalline solids soluble in ether, alcohol and dilute alkalis; some of their constants are shown in Table II.

TABLE II

Acid	Melting point	Yield per cent.	Analyses			
			Nitrogen		Sulphur	
			Found	Required	Found	Required
IV	145 to 146°C.	27	12.74	12.73	14.46	14.55
V	156 to 157°C.	41	8.96	8.97	20.28	20.26
VI	163 to 164°C.	44	11.31	11.29	12.87	12.90

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8. Liefert, *ibid.*, 1822, 114, 142.

DISCUSSION

The paper was read by Dr. Said.

The CHAIRMAN said that the Conference would welcome this contribution on synthetic work.

DR. HARTLEY (London) asked, firstly, if the introduction of the ethylsulphone group modified in any way the dissociation of the barbiturate portion of the molecule; that is, was the sodium salt formed readily hydrolysed or comparatively neutral? Secondly, if the introduction of

EFFECT OF THE SULPHONE GROUP IN HYPNOTICS

the ethyl-sulphone groups modified the way in which the molecule could be broken down by acid hydrolysis?

MR. D. E. SEYMOUR (Welwyn) asked what methods were used to make compound III. The combination of sulphones with the barbiturate would be a most difficult problem, and the compound probably would not have any value in this particular field. It was a sulphonamide, and might have quite different properties. Had Dr. Said examined his compounds for biological activity other than hypnotic value?

DR. K. BULLOCK (Manchester) asked if Dr. Said could tell them a little about the method of testing hypnotics. In relating constitution to physiological action, the method of testing was important. He asked whether the decreasing activity with increasing number of sulphone groups could be related to such properties as decreasing solubility, or change of pH.

DR. R. E. STUCKEY (London) said he could not quite follow Mr. Seymour's remark about compound III being essentially a sulphonamide, and would like that explained. It was interesting that the compound, with free hydrogen in position 5, showed no activity; with such a compound, the molecule was more acidic, and had a considerably lower K_A value.

DR. F. SAID, in reply, said that with one ethylsulphone group the compounds were more soluble in liquids than in water, and with two ethylsulphone groups the difference was even more marked. For testing the compounds dogs had been used, the relative activities being determined from the amounts needed to produce sleep. One group of dogs was given barbitone and another the compound to be tested, but he could not give details of the method as the test was done in the Pharmacological Department. Other biological activities had not been investigated. He agreed that it was desirable to investigate the germicidal activity, but doubted whether compound III could be described as a sulphonamide.

WOOL ALCOHOLS

PART I—OBSERVATIONS ON CHANGES IN PHYSICAL AND CHEMICAL PROPERTIES IN WOOL ALCOHOLS AS A RESULT OF OXIDATION

BY G. S. MUIRHEAD, K. H. OBERWEGER, D. E. SEYMOUR
AND D. SIMMONITE

(From the Research Department, Herts Pharmaceuticals Ltd.)

Received July 1, 1949

IN view of the increase in the use of wool alcohols* as an emulsifying agent in the preparation of pharmaceutical creams and ointments, and in particular, the inclusion of monographs on wool alcohols and ointments derived therefrom in the British Pharmacopoeia, it was considered that some results of investigations carried out in these laboratories on various aspects of the chemical and physical behaviour of wool alcohols would be of value to pharmaceutical workers.

A review of the literature indicates that very little information exists concerning the properties of wool alcohols and their behaviour when stored under different conditions. The results presented in this paper refer specifically to one particular grade of wool alcohols available in this country,† although experience gained by us suggests that wool alcohols produced in these and other associated laboratories differ in certain properties. It has been known for some considerable time that certain characteristic changes occur when wool alcohols are stored in air.^{1,2} For example, exposure of samples of the material to the atmosphere gives rise to a change in surface characteristics and suggestions have been made in the literature that this change is due to oxidation. Gillam,³ for example, reports that wool alcohols when exposed to atmospheric conditions, for considerable periods of time, show a marked increase in fatty acids, as denoted by rise in acid value, and it has been noted by us that wool alcohols, when subjected to irradiation with ultra-violet light showed marked changes in physical characteristics and rise in acid value. It has also been observed that these changes are slowed down by the addition of small quantities of antioxidants (e.g., pyrogallol, α -tocopherol). It has been our experience over several years that samples of wool alcohols when stored in bulk prior to use in a technical process, showed marked rises in acid value and such samples, on examination according to the B.P. monograph, possessed acid values as high as 30, and some samples contained negligible digitonin-precipitable material. It was also noted that such wool alcohols were unsuitable for use in the manufacture of water-in-oil emulsions.

Although we have shown that breakdown of water-in-oil emulsions is due to several factors other than the nature of the emulsifying agent itself (e.g. nature of hydrocarbon used, conditions employed in

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emulsification), it has been apparent that the initial nature of the wool alcohols or changes which they might have undergone during storage in the emulsion, may adversely affect the stability of the product. For example, a series of comparable emulsions prepared from wool alcohols of different origin showed marked differences in stability and those which tended to separate more readily showed a marked rise in acid value of the isolated oil phase, such rise being retarded by the inclusion of antioxidants.

In view of these observations it was considered that a quantitative study should be made of some of the physical and chemical properties of wool alcohols when treated as follows:—irradiation by means of ultra-violet light, exposure of the solid material to air at normal or elevated temperatures, and treatment of molten material with oxygen. This paper records some of the results obtained and the methods used. The changes in physical and chemical constants were measured by periodic determination of some or all* of the following:—

1. Surface activity; 2. Appearance; 3. Acid value; 4. Saponification value; 5. Acetyl value; 6. Digitonin-precipitable fraction.

EXPERIMENTAL

For convenience, the work will be described under the following sub-headings:—1. Treatment of molten wool alcohols with gaseous oxygen; 2. Exposure of wool alcohols to atmospheric oxygen; 3. Irradiation of wool alcohols with ultra-violet light; 4. Examination of wool alcohol emulsions after storage.

1. *Treatment of molten wool alcohols with gaseous oxygen.* The following method and equipment were devised with a view to producing a uniform rate of oxidation and causing appreciable changes in the material within reasonable periods of time. The apparatus is illustrated in Figure 1.

Oxygen enters at A and passes through the aperture B of the hollow glass stirrer G and partially through the stirrer guide C. It enters the molten wool alcohols through the holes D, E & F, in the stirrer and also through the base of the stirrer guide at H. Gas leaves the vessel at the side tube I and its flow rate was measured by a suitable meter. The lower portion of the apparatus was surrounded by a removable steam jacket so that test samples could be withdrawn via J. The stirrer speed was approximately 300 revolutions per minute. Samples for analysis were withdrawn at the intervals quoted and the results obtained are tabulated in Table 1. A control experiment was carried out substituting carbon dioxide for oxygen. In this way it was shown that negligible changes of the wool alcohols occurred in the absence of oxygen even on heating for the time quoted. As a further precaution the effluent gases were trapped in a wash bottle cooled with solid carbon dioxide so that any acidic volatile matter produced could be estimated by titration

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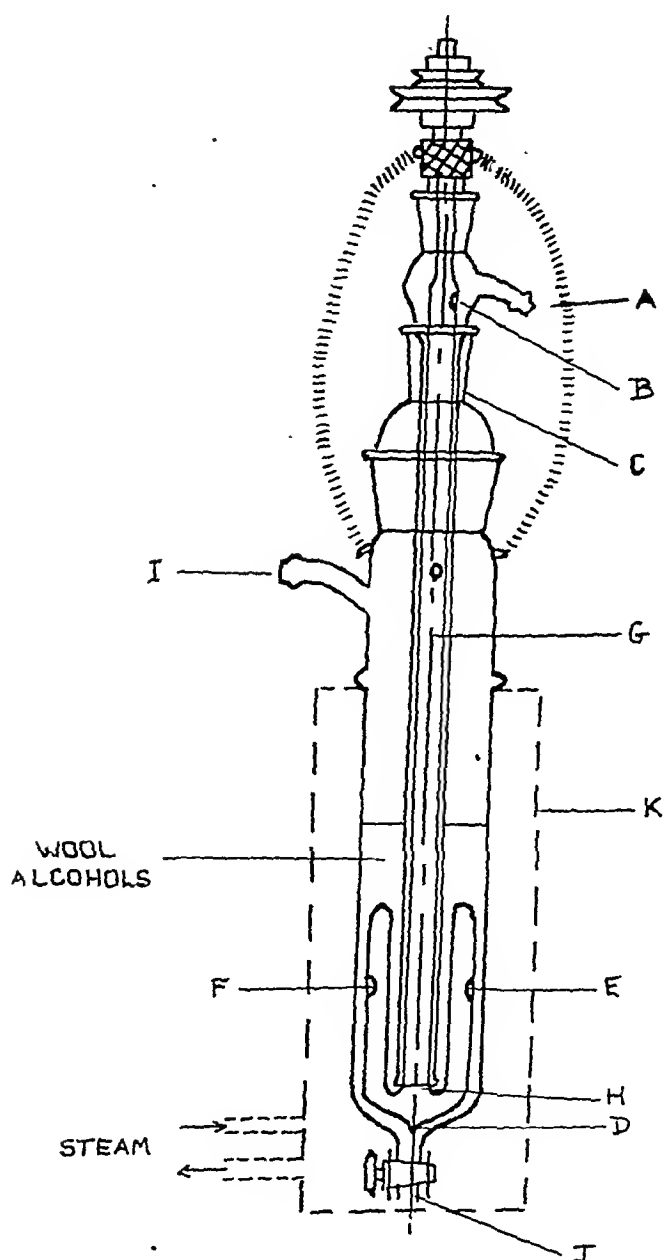


FIG. 1. Apparatus for the treatment of molten wool alcohols with gaseous oxygen in alcoholic solution. A small acidic fraction was obtained in this way which constituted approximately 1 per cent. of the total acidity and this was ignored in the acid values quoted in Table I.

2. Exposure of wool alcohols to atmospheric oxygen. The wool

WOOL ALCOHOLS. PART I

TABLE I

CHANGES IN CHEMICAL AND PHYSICAL PROPERTIES OF WOOL ALCOHOLS DURING OXIDATION

Time of oxygenation in hours	Acid value	Ester* value	Acetyl value	Cholesterol content (digitonide method) per cent.	Interfacial Tension Dynes/cm. Age of interface	
					60 secs.	120 secs.
0	2.5	4.7	134	30.40	8.5	7.4
5	4.0	23.4	132	25.49	10.6	9.6
10	5.3	24.1	122	23.85	—	9.2
20	8.1	32.5	—	20.43	8.1	7.2
30	12.0	38.6	110	15.14	7.6	6.5
40	16.0	61.2	—	8.32	6.1	4.7
50	25.4	62.0	—	4.99	4.3	3.9
60	31.1	78.6	86	0.81	4.5	3.7
70	34.0	96.0	—	—	5.2	4.5
80	35.3	96.7	55	—	4.3	4.0
90	38.4	96.6	—	—	5.1	4.4

* Difference between saponification value and acid value.

alcohols were obtained in suitably thin layers by pouring the molten material into large photographic developing dishes to a depth of 1/16 inch. The samples were then exposed to the atmosphere at the normal temperature and in an incubator at 37°C. Samples were withdrawn periodically and their acid values determined. The results are shown graphically in Figure 2.

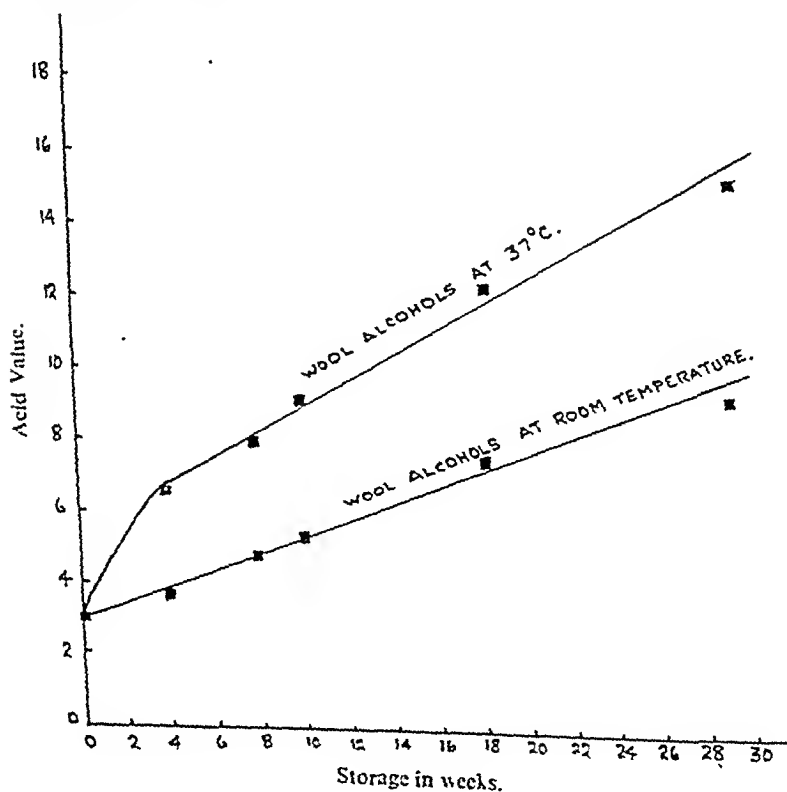


FIG. 2.

3. *Irradiation of wool alcohols with ultra-violet light.* The material was prepared in thin layers as in the previous experiment and exposed to the radiation of an Hanovia ultra-violet lamp, the quartz tube being approximately 8 inches from the surface of the wool alcohols. Samples were withdrawn at intervals and their acid values determined. The experiment was repeated using wool alcohols containing antioxidants. The results are shown graphically in Figure 3.

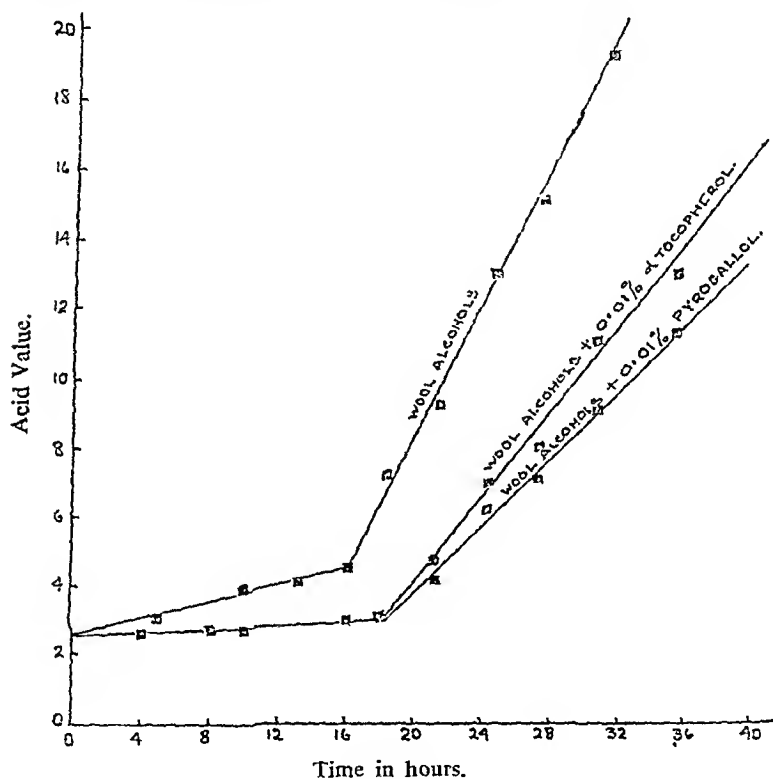


FIG. 3.

4. *Examination of wool alcohol emulsions after storage.* Two emulsions were prepared according to the following formula, one containing 0.007 per cent. w/w of propyl gallate.

Wool Alcohols	20
Paraffin wax	100
White oil (viscosity 74 Redwood secs.)	200
Water	680

The samples were packed in lacquered tinplate containers leaving a considerable air space above the emulsion and stored at room temperature and at 37°C. Samples were observed at various intervals of time, the appearance being noted and the acid value of the total oil phase determined by the following procedure:—A sample was withdrawn and twice its weight of warm water added. It was then heated on a steam bath until the emulsion had separated into distinct clear layers. The

oily layer was then separated and a suitable weight used for the determination of acid value as described later. It was assumed that only wool alcohols were responsible for any change in acid value of the oily phase and the value was calculated with reference to the known wool alcohol content. During the experiment some of the emulsions partially separated but the acid value determinations were nevertheless continued. The results are illustrated in Figure 4.

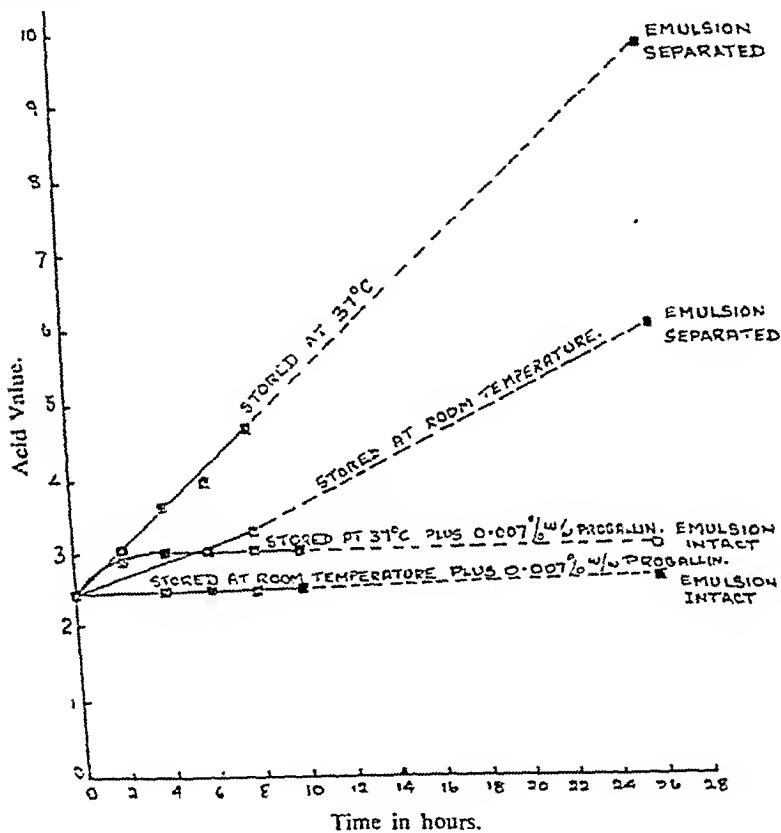


FIG. 4.

METHODS USED FOR THE DETERMINATION OF PHYSICAL AND CHEMICAL CONSTANTS

Acid value, cholesterol (digitonide method), and saponification value were determined by the method described in the British Pharmacopoeia 1948.

Acetyl value. The method described in the British Pharmacopoeia 1948, page 762, was found inconvenient for ordinary samples of wool alcohols and could not be applied at all to highly oxidised samples since the high viscosity of the acetylated material rendered its washing and recovery from the separating funnel extremely difficult. The following modification was therefore devised and proved satisfactory in use. After acetylation according to the B.P. method, the mixed acetates were

dissolved in chloroform, filtered and the extract washed with brine until neutral. After finally washing with water and drying over anhydrous sodium sulphate the solvent was removed. The determination was then completed in the normal manner.

Determination of surface activity by measurement of interfacial tension. Interfacial tension measurements were undertaken in an attempt to assess quantitatively any changes of emulsifying power which wool alcohols may have undergone as a result of oxidation. Heinrich,⁴ who well realised the usefulness of this tool for the study of cosmetic emulsions, made use of the pendant drop method for the measurement of interfacial tension. In addition to measurements on total wool alcohols, experiments with fractions were carried out.

The results are set out in Table II.

TABLE II
INTERFACIAL TENSION VALUES OF SOME WOOL ALCOHOL FRACTIONS

Fraction	Interfacial tension in dynes/cm. Age of interface 120 secs
Total wool alcohols (1 per cent. w/v in white oil*)	5.0
Cholesterol (1 per cent. w/v in white oil)	5.6
iso-Cholesterol (1 per cent. w/v in white oil) (Lanosterol-agnosterol fraction)	16.4
Cetyl alcohol (1 per cent. w/v in white oil)	13.8
Blank using water and white oil	52.5
Total wool alcohols (1 per cent. w/v in benzene)	7.4
Optically inactive alcohols (1 per cent. w/v in benzene)	12.4
Blank using water and benzene	32.2

* Viscosity 74 seconds Redwood

The possibilities of this method were extensively investigated by Andreas *et al.*⁵ whose techniques and calculations have been freely adopted in our work. Certain mathematical functions necessary for the application of their technique have recently been published in an improved form by Fordham⁶ whose tables have been used throughout this study.

The method consists of developing a suitably shaped drop of one phase beneath the surface of the other. From measurements of the drop aided by suitable magnification the interfacial tension of the particular system can be calculated. The apparatus is illustrated in Figure 5 and is substantially that used by Andreas *et al.*⁵. Certain features of difference will be described to supplement the diagram.

1. The lighting was obtained from a high power microscope lamp using 6 volt 8 amp. bulbs and fitted with adjustable lenses and an iris diaphragm.

2. The drops were expelled from a glass jet of suitable size by means of a 10 ml. hypodermic syringe fitted with a micrometer adjustment. A similar device had been used by Smith.⁷

3. The constant temperature cell was mounted together with the drop-forming mechanism in place of the stage of a microscope, the barrel being horizontal, so that multi-directional adjustment could be easily obtained. The camera was a Kodak $\frac{1}{4}$ plate, fitted with Compur shutter and ground glass focussing back. In practice it was found that suitable magnification to cover the different sizes of drops could be obtained by the use of 1 inch or $\frac{2}{3}$ inch microscope objectives.

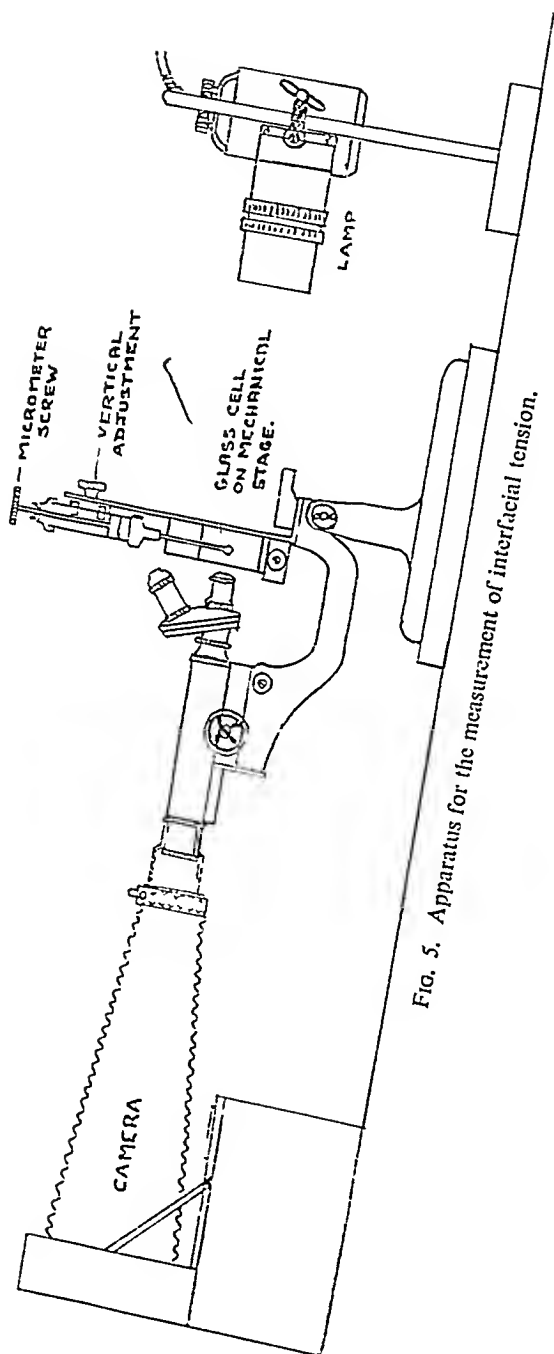
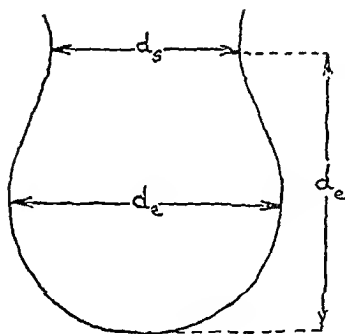


FIG. 5. Apparatus for the measurement of interfacial tension.

Briefly the experimental procedure was as follows :—The sample of wool alcohols under test was accurately weighed out dissolved in benzene* and adjusted to volume to give a 1 per cent. w/v solution. The solution was placed in the glass cell of the apparatus and drops of water contained in the syringe were developed on the end of a suitable jet as illustrated. The rate at which successive drops were formed was kept as uniform as possible and when the drop had the correct shape (possessed a suitable waist) it was photographed. The drop was photographed at intervals of 60 and 120 seconds after formation, since a finite time is necessary for the establishment of the interface and the attainment of a minimum value of interfacial tension. The plate was placed in a photographic enlarger and projected on to squared paper. The dimensions needed for the calculation were then measured directly and the interfacial tension calculated using the tables published by Fordham⁶ in conjunction with the following formula quoted by Andreas *et al.*⁵ :—

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where γ = interfacial tension in dynes per cm. between the two phases under consideration $\rho^1 - \rho^2$ = difference in densities of the two phases at the temperature of the experiment. g = acceleration due to gravity. H = function of S , which can be obtained from the HS functions for pendant drops given by Andreas *et al.*⁵ and Fordham.⁶ For a derivation of this relationship the original paper by Andreas *et al.* should be consulted.

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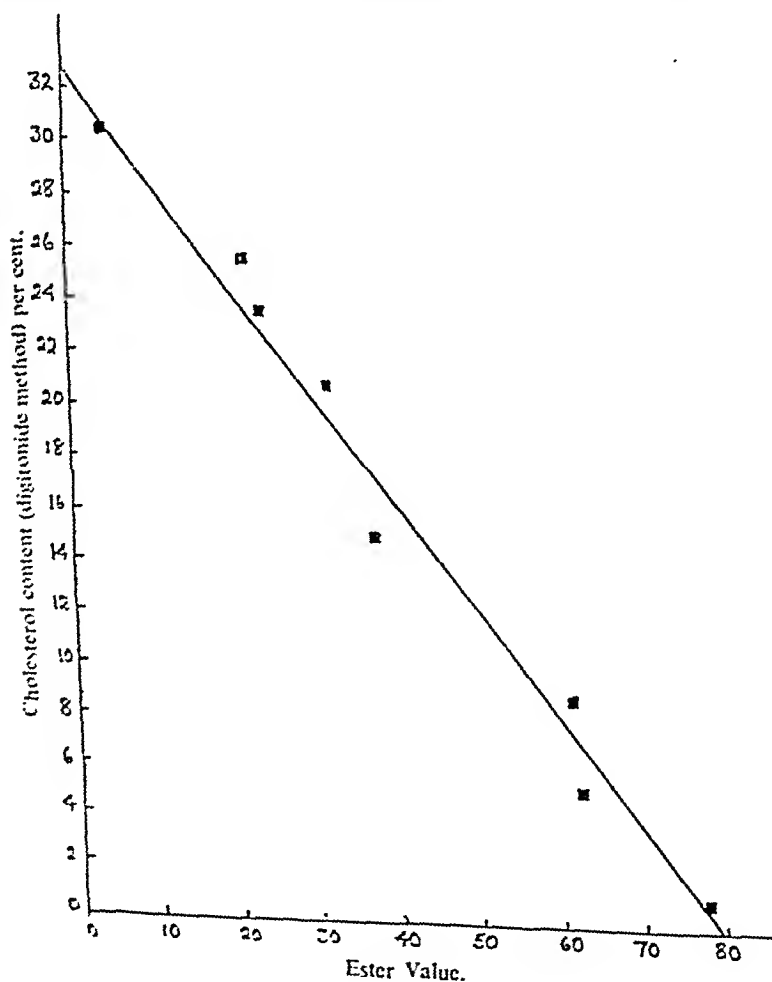
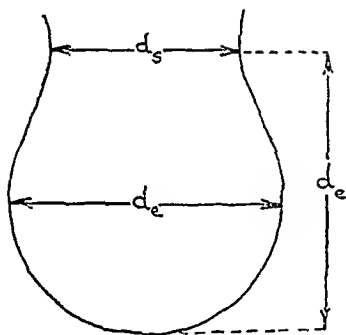


Fig. 6.

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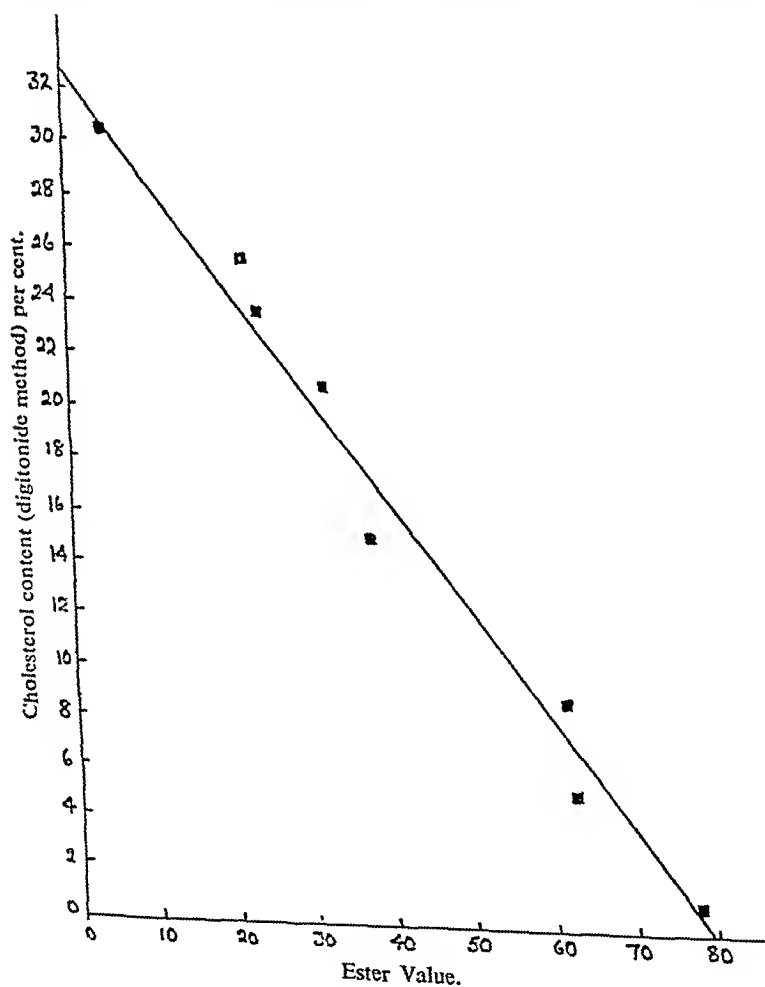
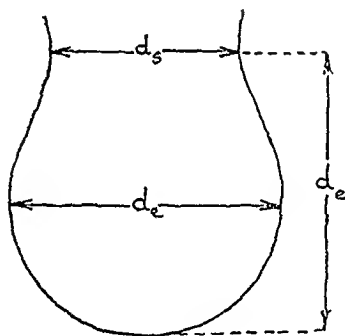


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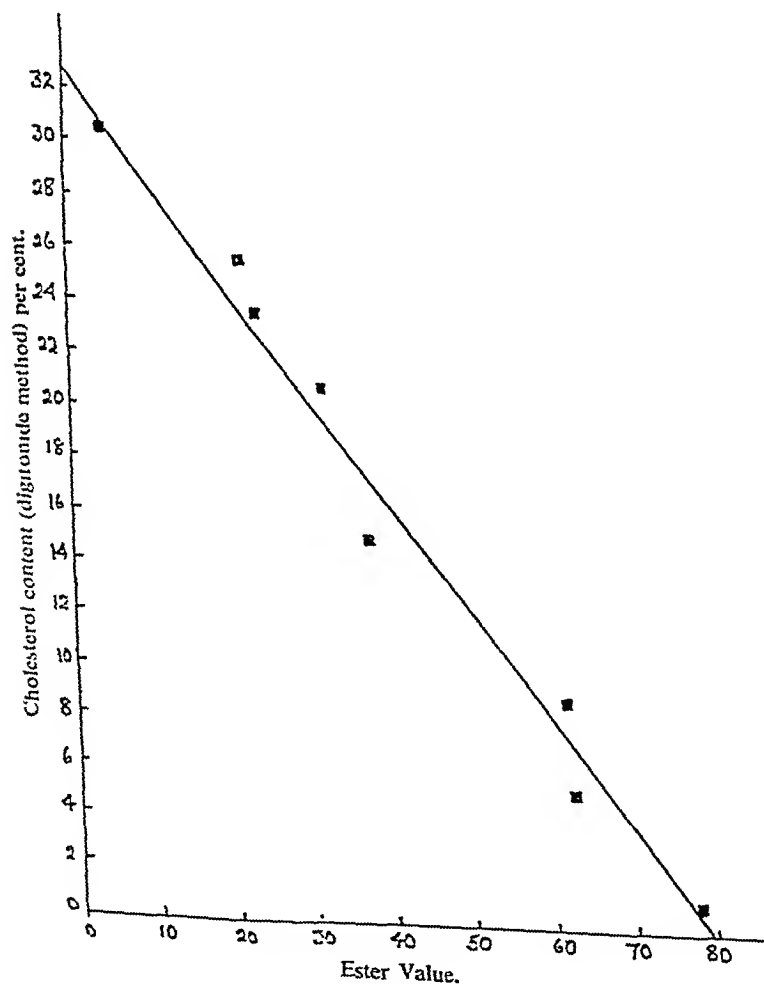


Fig. 6.

we have observed. It is interesting to note that the oxidised wool alcohols appear to show changes in physical properties which are suggestive of polymerisation, thus the material becomes increasingly vitreous and viscous, its melting point rises and its solubility in certain hydrocarbons decreases.

It is not proposed to elaborate however on possible mechanisms of degradation with the limited data which is available at this stage of the work. Further investigations on this subject will appear in subsequent communications.

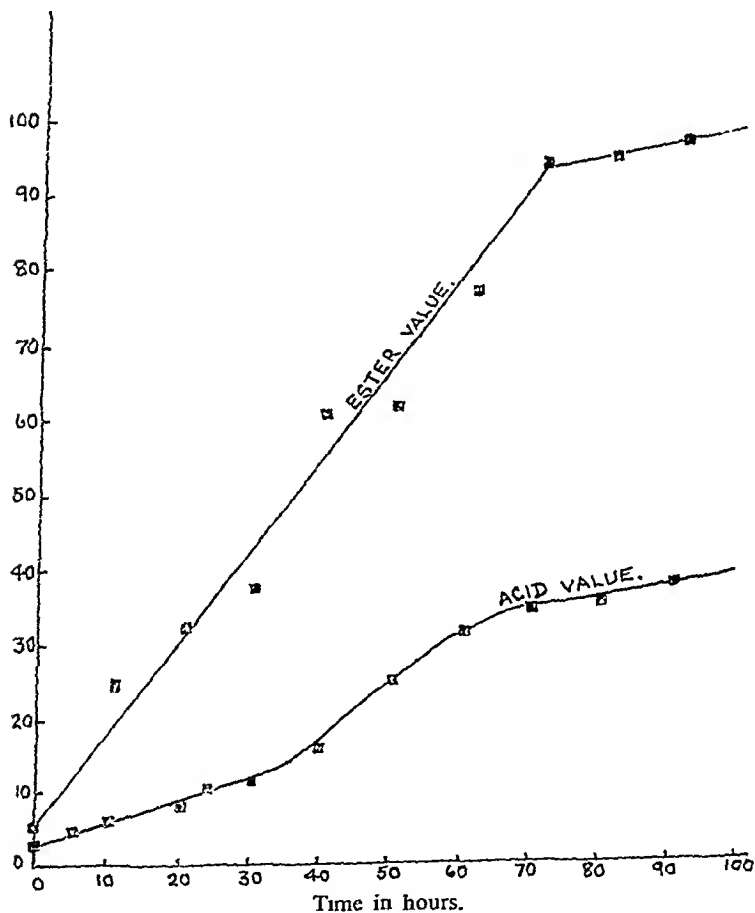


FIG. 7.

Interfacial tension measurements when carried out in a solvent in which samples at all stages of oxidation are completely soluble, show an initial fall with the attainment of a steady value when other chemical changes have become negligible. This suggests that wool alcohols do not suffer a deterioration of surface activity on oxidation. Their diminished efficiency as an emulgent may be attributable to loss of solubility in the hydrocarbon phase of the emulsion. The observations

concerning antioxidants are of interest and may be of practical value in the preservation of wool alcohols and emulsions prepared therefrom.

SUMMARY

1. Wool alcohols have been subjected to oxidation under varying conditions and progressive changes in physical and chemical properties have been observed. Such changes include—rise in acid and saponification value, decrease in acetyl value and cholesterol content.

2. The interfacial tension-reducing power of total wool alcohols and some isolated fractions have been measured using the pendant drop method. This value does not show any appreciable change as oxidation proceeds.

3. Wool alcohol emulsions suffer breakdown on storage. This is accompanied by rise in acid value of the oil phase. Both effects

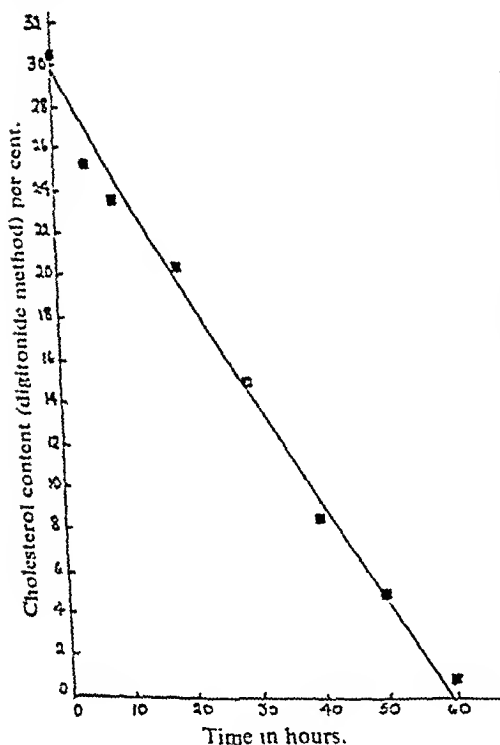


FIG. 8.

can be retarded by the inclusion of antioxidants in the formula.

4. Some tentative mechanisms based on the results obtained have been put forward to explain the changes which wool alcohols undergo on oxidation.

Much of the practical work was carried out by Miss E. Graydon, P. Hills and G. Sumpter. The authors wish to thank A. G. Wright for his work in connection with the design and construction of the two types of apparatus illustrated and the Directors of Herts Pharmaceuticals Limited for permission to publish this work.

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MR. J. H. OAKLEY (London) said that this work might partly explain why samples of hydrous ointment made on a larger scale were frequently

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alcohols, and the point about not having too much in stock at a time was very important. It should be stored in the dark and in sealed containers. It was very apparent if one looked at wool alcohols that there was an oxidised surface film, and material which they had had in stock for some time had failed to make an emulsion at all. On examining it afterwards they found that it had an acid value as high as 30; it was totally insoluble in the hydrocarbon phase. It was also important to keep it in large pieces and before use to scrape off the oxidised surface film containing the polymer.

They had recently commenced an investigation of penicillin ointment and were obtaining some surprising results, but they were not consistent.

Mr. Oakley's question about working on a large scale with wool alcohols was important. They had even considered working in an inert atmosphere, for example, under a blanket of carbon dioxide in the homogeniser, but had not yet adopted that procedure because of the technical problems which obviously arose. The possible effect of packing the material when the emulsion might be still warm had occurred to them.

Traces of metals might act as oxidation catalysts, and they were at the moment examining wool alcohols from different sources, to see what metals were present and what the effects might be on oxidation. The materials with which they had worked had been made in all-glass plant, and they did not get these problems so much with that type of material. They had not had any experience with chelating agents.

They had used the higher gallate esters because they were not soluble in water and had even considered cholesterol gallate. The lower esters were soluble in water and might cause darkening by contact with the iron of the plant. They understood that there was not much difference in efficacy between the lower esters and the higher ones, and ethyl gallate was unsuitable in a hydrous preparation because of its solubility in water.

Shallow containers were ideal for wool alcohol emulsions, though there was one great snag about them, namely, the very large surface area exposed to oxidation, which they now believed to be the main cause of breakdown.

MR. K. H. OBERWEGER, dealing with Dr. Bullock's point about the light phase, said they had put it down to an induction phase, which was not uncommon with natural materials of the type in question, possibly due to the presence of some natural antioxidant which would eventually be swamped by oxidation, and thus lose its activity.

THE OCCURRENCE OF METHYL COMPOUNDS IN GALENICALS

By H. E. BROOKES and H. K. JOHNSON

From the Analytical Laboratory, Boots Pure Drug Co., Ltd., Nottingham

Received July 9, 1949

THE presence of traces of methyl alcohol in the galenicals prepared from certain vegetable drugs has been previously recorded, and the fact is of considerable interest since the Board of Customs and Excise prohibits its presence in rebateable preparations. Richardson¹ showed evidence of such a possibility and found amounts varying from 0.01 to 0.10 per cent (0.045 to 0.48 per cent. based on alcohol content) in simple preparations of orange peel, gentian root, lemon peel, buchu and compound preparations of orange, gentian and rhubarb. In his opinion, the derivation of the colour in the reaction obtained in the British Pharmacopœia's modification of the Denigés test² was not the presence of essential oils or methyl esters, but of methyl alcohol itself, since adequate means were employed for removal of the former³. It was suggested that the methyl alcohol was derived from the decomposition of pectin present in orange and lemon peels. No observations on the technique of distillation were given.

Our interest in this matter was renewed recently, because of a complaint by the Customs and Excise Authorities with reference to a rebate claim on a production batch of concentrated compound infusion of gentian. They accepted that certain drugs on distillation may produce small amounts of methyl alcohol or substances giving similar reactions, but maintained that the proportion found, 0.21 per cent., was abnormally high and that the preparation must, therefore, be considered to contain industrial methylated spirit. Since methylated spirit was not used in the manufacture of the concentrated compound infusion of gentian, this and subsequent batches, made with pure alcohol, were tested for methyl alcohol and compared with samples of material marketed by other pharmaceutical houses. The test used was the B.P. test for the presence of methyl alcohol in alcohol which, as stated in Appendix XII G may give a positive response to the presence of methyl compounds as well as to methyl alcohol in the preparation. The former are converted to methyl alcohol in the test and the figures for methyl alcohol in this paper include that derived from such compounds. The results are recorded in Table I.

Methyl alcohol determinations on a number of batches of other galenicals prepared with pure rectified alcohol carried out as routine control, are recorded in Table II.

These results substantiated our contention that methyl compounds can be present in galenicals prepared according to the British Pharmacopœia and justified our intention to reinvestigate the original observations of Richardson, to show whether amounts of methyl compounds which the Customs and Excise Authorities had stated to be inadmissible were, in fact, liable to be present and if possible to determine the source or reasons for their production.

TABLE I

METHYL ALCOHOL IN CONCENTRATED COMPOUND INFUSION OF GENTIAN

Sample No	Alcohol content per cent	Methyl alcohol in galenical per cent	Methyl alcohol per cent of alcohol content
1	21.9	0.088	0.41
2	20.8	0.046	0.22
3	22.6	0.041	0.18
4	22.0	0.044	0.20
5	22.2	0.089	0.40
6	21.8	0.12	0.55
7	21.8	0.12	0.55
8	21.6	0.10	0.46
9	21.6	0.05	0.22
10	18.2	0.05	0.28
11	18.4	0.05	0.28
12	19.5	0.05	0.26
13	21.3	0.05	0.23
14	22.4	0.025	0.11
15	21.3	0.05	0.24
16	20.2	nil	nil
17	21.4	0.10	0.47

Samples numbered 1 to 5, 8 and 9 are taken from normal manufacturing batches. Those numbered 6 and 7 are from manufacturing batches made with previously disintegrated orange and lemon peels. Numbers 10 to 13 were made on a laboratory scale. Numbers 14 to 17 were materials purchased in the normal way from other drug houses.

TABLE II

METHYL ALCOHOL IN NORMAL BATCHES OF GALENICALS
(Carried out as routine control)

Preparation	Alcohol content per cent	Methyl alcohol in galenical per cent	Methyl alcohol per cent of alcohol content
Ammoniated tincture of valerian	50.1	0.10	0.20
Compound tincture of gentian	41.2	0.008	0.019
Concentrated compound tincture of gentian B.P. 1932, 5th addendum	35.2	0.008	0.023
Camphorated tincture of opium	58.0	0.0025	0.004
Tincture of belladonna	68.4	0.0055	0.008
Tincture of ipecacuanha	23.6	nil	nil
Tincture of orange	73.6	0.007	0.009
Tincture of squill	55.2	nil	nil
Tincture of calumba	58.4	nil	nil
Tincture of lemon (for syrup of lemon)	34.4	0.11	0.32
Tincture of hyoscyamus	66.4	0.007	0.011
Strong tincture of ginger	82.8	0.008	0.01
Tincture of aloes	39.6	0.008	0.02
Tincture of opium	42.8	0.008	0.019
Compound tincture of rhubarb	50.0	0.005	0.01
Tincture of serpentary	59.0	0.006	0.01
Tincture of myrrh	84.8	0.02	0.024
Tincture of strophanthus	67.6	0.001	0.001
Liquid extract of senega	39.6	0.44	1.1
Liquid extract of cascara	22.4	nil	nil
Liquid extract of liquorice	17.8	nil	nil
Liquid extract of ergot	48.4	0.005	0.010
Liquid extract of sarsaparilla	13.2	0.004	0.030
Concentrated infusion of orange peel	21.2	0.12	0.57
" " " "	22.0	0.12	0.55
" " " "	22.0	0.08	0.36
Concentrated infusion of valerian	23.0	0.033	0.14
Eluxir of senna	12.8	0.08	0.62
Concentrated compound decoction of sarsaparilla	21.0	0.0018	0.009

EXPERIMENTAL

The alcohol content was obtained (a) by the British Pharmacopœia method or (b) by direct distillation; where ammonia was present, the distillate was neutralised to solid phenolphthalein and redistilled.

The method of the British Pharmacopœia for detection of methyl

METHYL COMPOUNDS IN GALENICALS

alcohol was adapted to quantitative use by comparison against controls as follows:

An amount of the distillate obtained by method (a) or (b) calculated by preliminary experiment to contain the equivalent of between 0.0001 ml. and 0.001 ml. of methyl alcohol was taken, sufficient alcohol added to produce finally a 10 per cent. solution, and diluted to 5 ml. with water. The pharmacopœial test for methyl alcohol was carried out on this solution and controls containing 0.0001, 0.00015, 0.0002, etc., ml. of methyl alcohol in 5 ml. of 10 per cent. methyl alcohol-free ethyl alcohol, and the colour produced compared.

All alcohol used in the experiment and on production tests was checked for methyl alcohol content, and all results recorded were corrected for the trace of methyl alcohol present in the ethyl alcohol used. The results given in Tables I and II were obtained on distillates obtained by the British Pharmacopœial method.

In order to account for the high methyl alcohol content of liquid extract of senega recorded in Table II, a batch was examined at each stage of manufacture. The alcohol was determined by the method of the British Pharmacopœia (a) and by direct distillation (b), the tests being carried out on the distillates. The methyl alcohol content of the menstruum was not detectable by the method of the British Pharmacopœia. The results are given in Table III.

TABLE III

METHYL ALCOHOL FOUND DURING THE MANUFACTURE OF LIQUID EXTRACT OF SENEGA

	Methyl alcohol by method (a) per cent.	Methyl alcohol by method (b) per cent.
Reserve portion of the percolate	0.28	0.01
Soft extract from the remainder of the percolate	0.74	0.02
Liquid extract of senega	1.40	0.01

The above results indicate that the methyl alcohol obtained was produced during the assay. To confirm this, water, ethyl alcohol and an excess of sulphuric acid were added to the residue from the distillation of the liquid extract by method (b), and a normal distillation performed. The methyl alcohol content of the distillate was 0.52 per cent. calculated to the liquid extract. Since this was insufficient to account for the high methyl alcohol content of the final product, this process was repeated, starting with a distillation from the liquid extract by method (b). When the acid, alcohol and water had been added to the residue, it was distilled very slowly. The distillate gave 0.88 per cent. methyl alcohol calculated to the liquid extract, a quantity greater than the previous figure, but less than that obtained for the galenical. A further addition of water and alcohol to the residue was again slowly distilled. The methyl alcohol content of the distillate was 0.48 per cent. calculated to the liquid extract. The sum of the percentages of methyl alcohol obtained from the residue is 1.36, an amount comparable with that given for the finished product in Table III. This shows that the length of time taken in the distillation

by the method of the British Pharmacopœia affects the amount of methyl alcohol formed, and, further, that its source is undoubtedly the senega.

A production batch of concentrated infusion of senega B.P. gave methyl alcohol when the method of the British Pharmacopœia for alcohol content by acid distillation was used, to the extent of 0.36 per cent., whereas by direct distillation the amount was only 0.04 per cent. This latter figure was higher than expected and suggested that some alkaline hydrolysis occurred during preparation.

Owing to our Tinctures Department finding the recovered alcohol from the preparation of this galenical to be contaminated with methyl alcohol, the process was examined in detail, using the same drugs. The schematic diagram indicates the method of production, points of control testing and amounts of methyl alcohol found at each stage of production.

The method of preparation used in our Tinctures Department differs from that described in the British Pharmacopœia by the use of ammonia in the percolation, the modification aiding preparation and giving a better final product. That this small deviation from the British Pharmacopœia directions does not affect the findings was shown by the results of testing a sample prepared strictly by the British Pharmacopœia procedure, in which closely similar figures for the final methyl alcohol content were obtained.

In conclusion we determined the methyl alcohol in the distillate from a mixture of powdered senega, pure ethyl alcohol and water:

	<i>per cent. methyl alcohol calc. to senega</i>
1. Direct distillation, adding no acid or alkali	0.10
2. Distilled in presence of acid	1.20
3. Distilled in presence of ammonia, neutralised and redistilled	0.80

Hence it is demonstrated that for senega root and its galenical preparations, a small amount of preformed methyl alcohol may be present, but alkaline or acid distillation produces considerable amounts probably due to hydrolysis, acid giving the greater. The presence of methyl alcohol was confirmed by the Riche and Bardy⁴ test.

The presence of methyl salicylate in senega to an extent of 0.25 per cent has been recorded^{5,6}. Distillation of small amounts of methyl salicylate with dilute alcohol and water showed that ammonia will hydrolyse this ester completely in dilute alcohol, but dilute acid has no effect.

Richardson¹ suggested that the methyl alcohol might be derived from the hydrolysis of pectin and the gelatinisation of senega preparations has been attributed to the occurrence of pectinous substances in the drug^{6,7}. By distillation of pectin far in excess of the proportions likely to be present in the galenicals tested, some methyl alcohol was obtained, the

METHYL COMPOUNDS IN GALENICALS

amount from acid distillation being comparable with that from alkaline distillation. Pectin should not be present in senega preparations to any extent, since alkaline distillation of senega produces much less methyl alcohol than acid. This suggests other substances to be the main source of the methyl alcohol.

In our opinion, the production of methyl alcohol by hydrolysis during distillation may not necessarily always account for its presence in galenicals. In support of this, we can quote some experiments on concentrated infusion of orange which was examined at all stages of manufacture, showing relatively high proportions of methyl alcohol on each test using the method of the British Pharmacopœia for alcohol determination (which does not require acid distillation and eliminates esters by light petroleum extraction of the distillate) but also showing similar results by a direct neutral distillation. These results suggest that in preparations of orange, the methyl alcohol may occur in the orange peel.

CONCLUSIONS

Based on our work with senega and the results given by routine examinations of other galenicals, it may be inferred that methyl compounds may occur in many galenicals in more than traces and that some of these may be hydrolysed to methyl alcohol. Since acid or alkaline media increase this hydrolysis, it would be desirable for the test for methyl alcohol to be made on a distillate from neutral solution. As considerable frothing impedes distillation of many galenicals, especially senega, unless acid is present, a correct alcohol content should be determined by the method of the British Pharmacopœia, and the methyl alcohol test carried out separately, distilling from neutral solution.

SUMMARY

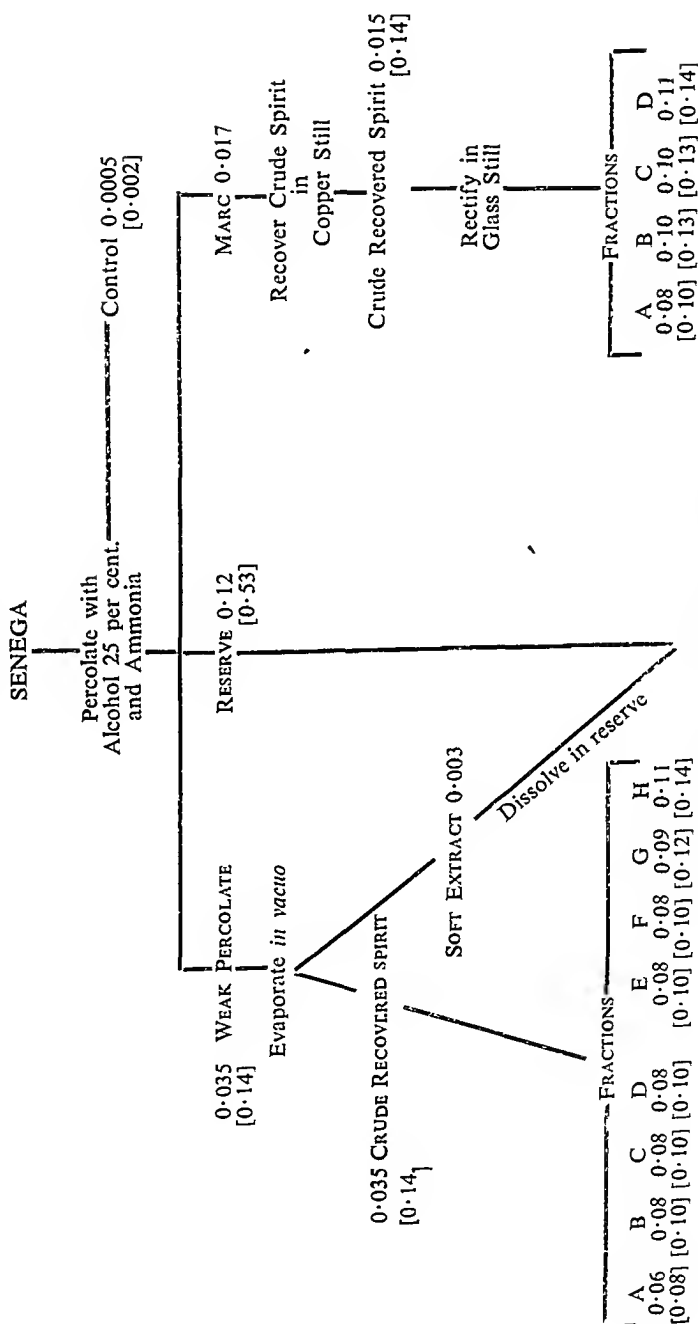
1. The presence of methyl alcohol in galenicals in more than traces is recorded and previous observations confirmed.
2. The source of the methyl alcohol in senega preparations has been closely investigated.
3. The method of the British Pharmacopœia for alcohol content has been shown to be the main contributory cause of the production of methyl alcohol by acid hydrolysis of the senega extractives.
4. In these and other galenicals some preformed methyl alcohol is present.

We wish to express our thanks to Mr. D. A. Hughes for his co-operation in the preparation of samples for examination, and to the Directors of Boots Pure Drug Co., Ltd., for permission to publish this work.

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CONCENTRATED INFUSION OF SENEGA B.P.



DISCUSSION

The paper was read by Mr. H. E. Brookes.

The CHAIRMAN said he thought most people who dealt with galenicals had been aware that traces of methyl alcohol were frequently found when no methyl alcohol had been used in their preparation, particularly in the case of senega. A most interesting observation in this paper was the fact that the acid distillation used in the B.P. assay increased the amount of methyl alcohol in the case of senega.

DR. G. E. FOSTER (Dartford) said that he had also met this problem of methyl alcohol in galenicals. The Customs and Excise Authorities would not allow a rebate in certain cases, although only pure ethyl alcohol had been used. If anyone implied that methylated spirit had been used, it was up to them to prove it, and he did not think they would be able to prove it against the manufacturing records that could be produced.

DR. W. MITCHELL (London) said that these results would be welcomed, not only by manufacturers, but by the Government Chemist's Department. The Customs and Excise Department were not so accommodating as Dr. Foster had suggested, and the line they always took was that no manufacturing records could cover the possibility of accidental mixing of a small amount of industrial spirit with S.V.R. The Customs and Excise Authorities had complained to his Company that a sample of concentrated infusion of senega contained an excessive amount of methyl alcohol. After much correspondence the Authorities had accepted the Company's figure of 0.1 per cent., but had said that the maximum they could allow was 0.03 per cent. and anything in excess of that must have been added, accidentally or deliberately. They had admitted that they did not suspect the Company's bona fides, but were tied by the literature. The Authorities had further said that if evidence could be provided to support the claim that infusion of senega could contain 0.1 per cent. or more they would consider it. In his laboratory they had recently tested many batches of senega preparations, and so far had not found any containing as much as 0.1 per cent. of methyl alcohol. He was interested that the present authors had found these higher figures, and he asked how much methyl alcohol they had found in their alcohol. Had they used the chromotropic acid test as a confirmatory test? He had found it satisfactory in confirming the B.P. test.

MR. BROOKES, in reply, said that they also had obtained very little redress from the Customs and Excise Authorities. He was pleased to have the figure of the maximum amount of methyl alcohol allowed as 0.03 per cent. By the usual Schiffs reagent method it was difficult to determine the content of methyl alcohol in alcohol, but they had managed to get some very good Schiffs reagent and found it to be 0.0005 per cent. of methyl alcohol. He had not used the chromotropic acid test.

PARA-AMINOSALICYLIC ACID—PART III

SOME FURTHER STUDIES ON THE *IN VITRO* TUBERCULOSTATIC BEHAVIOUR OF PARA-AMINOSALICYLIC ACID AND RELATED COMPOUNDS

BY D. J. DRAIN, C. L. GOODACRE AND D. E. SEYMOUR

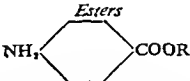
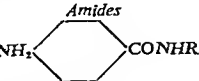
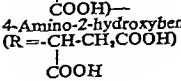
From the Research Department, Herts Pharmaceuticals, Ltd.

Received June 23, 1949

In our previous report¹, some preliminary results showed that simple substitution in the *p*-aminosalicylic acid molecule did not produce any compound possessing an *in vitro* tuberculostatic activity markedly higher when using *Mycobacterium tuberculosis* H37RV strain. This paper is concerned with the *in vitro* study of a wider range of 1-2-4-tri-substituted aromatic compounds, the majority of which have not been previously described; their chemistry will be reported upon elsewhere. The culture medium used throughout the work and the technique for the determination of activity was similar to that described in our earlier paper¹. It may be of interest to note that we have confirmed the observations made by Youmans *et al.*² that the presence of tween 80 (polyoxyethylene sorbitan mono-oleate) in the medium markedly influences the tuberculostatic behaviour of *p*-aminosalicylic acid and other compounds; this

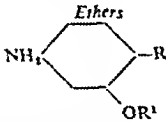
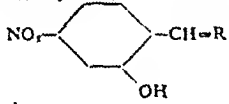
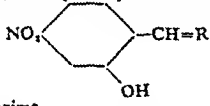
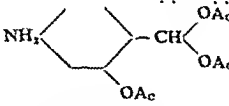
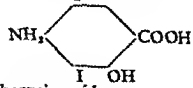
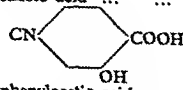
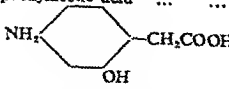
TABLE I

THE INHIBITORY CONCENTRATION OF *p*-AMINOSALICYLIC ACID AND RELATED COMPOUNDS. INOCULUM OF 0.001 MG/ML. OF THE H37RV STRAIN

Standards							Inhibitory concentration mg. 100 ml. (after 14 days at 37° C.)
GROUP A							
1	<i>p</i> -Aminosalicylic acid	0.0487—0.0243
2	Streptomycin	0.0121—0.006
GROUP B							
		<div style="text-align: center;"> <i>Esters</i>  </div>					
3	β-Diethylaminoethyl-4-amino-2-hydroxybenzoate (R = -CH ₂ .CH ₂ .N (C ₂ H ₅) ₂)	0.1 — 0.01 (0.195 — 0.0975)
4	β-Hydroxyethyl-4-amino-2-hydroxybenzoate (R = -CH ₂ .CH ₂ .OH)	100—10
GROUP C							
		<div style="text-align: center;"> <i>Amides</i>  </div>					
5	4-Amino-2-hydroxybenzanilide (R = -C ₆ H ₅)	10—1
6	4-Amino-2-hydroxybenz- <i>p</i> -toluidide (R = -C ₆ H ₄ .CH ₃)	10—1
7	4-Amino-2-hydroxyhippuric acid (R = -CH ₂ .COOH)	100—10
8	4-Amino-2-hydroxybenzoyl alanine (R = -CH ₂ .CH ₃)	10—1
9	<div style="text-align: center;">  </div> 4-Amino-2-hydroxybenzoyl-DL-aspartic acid (R = -CH ₂ .CH(COOH).COOH)	100—10

PARA-AMINOSALICYLIC ACID. PART III

TABLE I—continued

Standards					Inhibition concentration mc. per 100 ml. (after 14 days at 37°C.)
GROUP C—cont.					
10	4-Amino-2-hydroxybenzoic acid hydrazide (R=NH ₂)	0.1—0.01 (0.0075—0.0487)
GROUP D					
					
11	* 4-Amino-2-methoxybenzoic acid (R=COOH, R'=CH ₃)	> 100
12	4-Amino-2-methoxytoluene hydrochloride (R=CH ₃ , R'=CH ₃)	100—10
13	4-Amino-2-ethoxytoluene hydrochloride... (R=CH ₃ , R'=C ₂ H ₅)	100—10
14	4-Amino-2-n-propoxytoluene hydrochloride (R=CH ₃ , R'=CH ₂ CH ₂ CH ₃)	100—10
15	4-Amino-2-n-butoxytoluene hydrochloride (R=CH ₃ , R'=CH ₂ (CH ₂) ₃ CH ₃)	100—10
GROUP E					
	4-Nitrosalicylaldehyde derivatives				
					
16	4-Nitrosalicylaldehyde (R=O)	1—0.1
17	4-Nitrosalicylaldehyde oxime (R=N.OH)	10—1
18	4-Nitrosalicylaldehyde semicarbazone (R=N.NHCONH ₂)	10—1
19	4-Nitrosalicylaldehyde thiosemicarbazone (R=N.NHCSNH ₂)	1—0.1
GROUP F					
	4-Aminosalicylaldehyde derivatives				
					
20	4-Aminosalicylaldehyde oxime (R=N.OH)	10—1
21	4-Aminosalicylaldehyde semicarbazone	0.01 — 0.001 (0.003 — 0.0015)
22	4-Aminosalicylaldehyde thiosemicarbazone	10—1
					
GROUP G					
	Miscellaneous				
23	3,5-diiodo-4-amino-2-hydroxybenzoic acid 	0.1—0.01 (0.0075—0.0487)
24	4-cyano-2-hydroxybenzoic acid 	100—10
25	4-Amino-2-hydroxyphenylacetic acid 	> 100

* Youmans, Raleigh, and Youmans⁷ reported that this compound does not inhibit growth of 0.01mg/ml of *M. tuberculosis* H37RV at a concentration of 10 mg/100 ml.

substance was, however, retained in our medium as its presence facilitates the turbidimetric standardisation of inoculum. The less soluble compounds were dissolved in propylene glycol, which was found to have no tuberculostatic effect when used in concentrations of 1 per cent. and below.

Table I shows the inhibitory concentrations of the compounds examined when using a standard inoculum of 0.001 mg./ml. (dry bacterial substance) of *M. tuberculosis* H37RV strain. The standards used were *p*-aminosalicylic acid and streptomycin and compounds exhibiting activities of an order similar to the two standards were examined in closer dilutions.

The above results coupled with those obtained by others^{3,4,5,6,7,8} suggest that there is no apparent relationship between chemical structure and *in vitro* tuberculostatic activity in this group of compounds, and from data obtained it would appear difficult to predict the effect of a simple variation in structure on *in vitro* activity. One can conclude that there is a high degree of specificity of the *p*-aminosalicylic acid molecule for tuberculostatic activity, and with the possible exception of the esters, alteration in molecular structure gives rise to markedly diminished activity. The anomalous results relating to the activity of the esters^{1,4,5,6,7} may be due to their low solubility and tendency to hydrolyse under conditions of test. The effect of nuclear substitution with the exception of halogens has received no attention, and we propose to investigate this type of compound and report more fully.

We are of the opinion, however, that some of the compounds referred to in our previous paper, together with compounds No. 3,4,7 and 10, are worthy of a preliminary *in vivo* examination in view of the possibility that they may possess certain advantages over *p*-aminosalicylic acid by being less rapidly absorbed and excreted. The aldehyde derivatives (Groups E and F) have special interest for animal work in view of the reports by Domagk *et al.*⁹ that certain benzaldehyde thiosemicarbazone derivatives have promising properties in the treatment of some forms of tuberculosis. Compound No. 21 (the thiosemicarbazone of *p*-aminosalicylaldehyde) is considered to possess sufficiently high *in vitro* activity to justify a trial in animals. For comparison purposes we report in Table II the *in vitro* activities of two of the compounds studied by

TABLE II

		Inhibition concentration mg./100 ml.
26	<i>p</i> -methoxybenzaldehyde thiosemicarbazone $\text{CH}_3\text{O}-\text{C}_6\text{H}_4-\text{CH}=\text{N.NH.CS.NH}_2$	0.0487—0.0243
27	<i>p</i> -acetylaminobenzaldehyde thiosemicarbazone $\text{CH}_3\text{CONH}-\text{C}_6\text{H}_4-\text{CH}=\text{N.NH.CS.NH}_2$	0.0975—0.0487

Domagk, namely, *p*-methoxybenzaldehyde thiosemicarbazone and *p*-acetylaminobenzaldehyde thiosemicarbazone.

The observation that the action of *p*-aminosalicylic acid is antagonised by *p*-aminobenzoic acid^{1,7} has led us to study the possible effects of other members of the vitamin B group; at this stage it is possible to report that such antagonism is not displayed by pteroylglutamic acid. The significance of this cannot be realised until further results are obtained. It is interesting to note that the activity of sulphathiazole against *Staphylococcus aureus*¹⁰ is similarly antagonised by *p*-aminobenzoic acid and not by folic acid.

SUMMARY

1. A series of tri-substituted aromatic compounds have been synthesised and their *in vitro* activities against the tubercle bacillus determined. The results of the study indicate no apparent relationship between structure and activity.

2. The thiosemicarbazone of *p*-aminosalicylaldehyde has been synthesised and its activity compared with the thiosemicarbazones of *p*-acetylaminobenzaldehyde and *p*-methoxybenzaldehyde.

The authors wish to thank Mr. D. Suddaby, B.Sc., A.R.J.C., and Mr. B. W. Mitchell, B.A. (Cantab.), A.R.I.C., for the preparation of some of the compounds and the Directors of Herts Pharmaceuticals Limited for permission to publish these results.

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substance was, however, retained in our medium as its presence facilitates the turbidimetric standardisation of inoculum. The less soluble compounds were dissolved in propylene glycol, which was found to have no tuberculostatic effect when used in concentrations of 1 per cent. and below.

Table I shows the inhibitory concentrations of the compounds examined when using a standard inoculum of 0.001 mg./ml. (dry bacterial substance) of *M. tuberculosis* H37RV strain. The standards used were *p*-aminosalicylic acid and streptomycin and compounds exhibiting activities of an order similar to the two standards were examined in closer dilutions.

The above results coupled with those obtained by others^{3,4,5,6,7,8} suggest that there is no apparent relationship between chemical structure and *in vitro* tuberculostatic activity in this group of compounds, and from data obtained it would appear difficult to predict the effect of a simple variation in structure on *in vitro* activity. One can conclude that there is a high degree of specificity of the *p*-aminosalicylic acid molecule for tuberculostatic activity, and with the possible exception of the esters, alteration in molecular structure gives rise to markedly diminished activity. The anomalous results relating to the activity of the esters^{1,4,5,6,7} may be due to their low solubility and tendency to hydrolyse under conditions of test. The effect of nuclear substitution with the exception of halogens has received no attention, and we propose to investigate this type of compound and report more fully.

We are of the opinion, however, that some of the compounds referred to in our previous paper, together with compounds No. 3,4,7 and 10, are worthy of a preliminary *in vivo* examination in view of the possibility that they may possess certain advantages over *p*-aminosalicylic acid by being less rapidly absorbed and excreted. The aldehyde derivatives (Groups E and F) have special interest for animal work in view of the reports by Domagk *et al.*⁹ that certain benzaldehyde thiosemicarbazone derivatives have promising properties in the treatment of some forms of tuberculosis. Compound No. 21 (the thiosemicarbazone of *p*-aminosalicylaldehyde) is considered to possess sufficiently high *in vitro* activity to justify a trial in animals. For comparison purposes we report in Table II the *in vitro* activities of two of the compounds studied by

TABLE II

		Inhibition concentration mg./100 ml.
26	<i>p</i> -methoxybenzaldehyde thiosemicarbazone $\text{CH}_3\text{O}-\text{C}_6\text{H}_4-\text{CH}=\text{N.NH.CS.NH}_2$	0.0487—0.0243
27	<i>p</i> -acetylaminobenzaldehyde thiosemicarbazone $\text{CH}_3\text{CONH}-\text{C}_6\text{H}_4-\text{CH}=\text{N.NH.CS.NH}_2$	0.0975—0.0487

PARA-AMINOSALICYLIC ACID. PART IV

Lehmann², Hurni¹, and Seivers³ suggest that "drug-fastness" is not a significant problem in the treatment of tuberculosis with this drug.

Whilst this paper was in course of preparation, Graessle and Pietrowski⁵ reported that repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 120 days failed to produce an increase in the resistance of the strain.

TABLE I

Case No	Before treatment	<i>p</i> -Aminosalicylic Acid treatment		
		2 months	3 months	4 months
1	0 0243—0 0121			0 0975—0 0457
2	0 0457—0 0243	0 0243—0 0121		
3	0 0243—0 0121			0 0457—0 0243
4	0 0243—0 0121			0 0457—0 0243
5	0 0243—0 0121			0 0457—0 0243
6	0 0243—0 0121			0 0243—0 0121
7	0 0457—0 0243	0 0457—0 0243		0 0457—0 0243
8	0 0457—0 0243	0 0457—0 0243		0 0457—0 0243
9	0 0243—0 0121			0 125 —1 56
10	0 0243—0 0121	0 0243—0 0121		0 0121—0 006
11	0 0457—0 0243	0 0457—0 0243		0 0457—0 0243
12	0 0457—0 0243	0 0243—0 0121		0 0457—0 0243
13	0 39 —0 195	0 39 —0 195		0 0975—0 0457
14	0 0457—0 0243	0 0457—0 0243		0 0457—0 0243
15	0 0243—0 0121	0 0243—0 0121		0 0243—0 0121
16	0 0243—0 0121			0 0243—0 0121
17	0 0457—0 0243		0 0457—0 0243	
18	0 0457—0 0243		0 0457—0 0243	
19	0 0243—0 0121	0 0243—0 0121		
20	0 0243—0 0121	0 0243—0 0121		
21	0 0457—0 0243			0 0457—0 0243
22	0 0243—0 0121			0 0243—0 0121
23	0 0243—0 0121	0 0243—0 0121		
24	0 0243—0 0121	0 0243—0 0121		
25	0 0457—0 0243		0 0457—0 0243	

The authors are indebted to Dr. R. Shoulman of the Highlands Hospital, Winchmore Hill, and Dr. J. Alston of the Archway Hospital, N.19, for the supply of the cultures. They also wish to thank the Directors of Herts Pharmaceuticals Ltd., for permission to publish.

SUMMARY

1. After repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 10 months, no increase in resistance developed.
2. Out of a total of 25 strains of *M. tuberculosis* isolated from patients receiving *p*-aminosalicylic acid, only one developed any increase in resistance after four months' treatment.

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PARA-AMINOSALICYLIC ACID—PART IV

ATTEMPTS TO INDUCE RESISTANCE TO PARA-AMINOSALICYLIC ACID, IN STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

BY C. L. GOODACRE AND D. E. SEYMOUR

From the Research Department, Herts Pharmaceuticals Ltd.

Received, June 23, 1949

THE unfortunate limitation of streptomycin in the treatment of tuberculosis, due to the development of drug resistance, suggested to us the necessity for an investigation as to whether similar phenomena might occur with *p*-aminosalicylic acid.

The investigation was concerned with attempts to induce drug-resistance *in vitro*, and with a study of strains of *M. tuberculosis* isolated from patients before and during *p*-aminosalicylic acid treatment. The H37RV strain was cultivated in Dubos medium containing decreasing amounts of the acid. After a suitable incubation period, the bacilli in the tubes containing the highest concentration of *p*-aminosalicylic acid which was still allowing growth were freed from it by washing, and used to inoculate a similar series of tubes. No increased resistance was demonstrated by this method, in fact, no growth at all occurred at the dilutions used after two or more passages. These results are confirmed by Hurni¹ who reported on a similar study while this work was in progress. In view of the unsatisfactory results obtained with the above method, the following procedure was adopted in an attempt to induce drug resistance. A large inoculum (0.5 mg./ml. of dry bacterial substance) of *M. tuberculosis* H37RV was introduced into Dubos medium containing *p*-aminosalicylic acid in a concentration of 100 mg./ml. After 14 days' incubation, a similar concentration of *p*-aminosalicylic acid in Dubos medium was inoculated from this culture. Inhibition concentration tests were made at monthly intervals, and after 10 months (20 passages) the organisms showed a similar *p*-aminosalicylic acid sensitivity to that at the beginning of the experiment. A duplicate experiment was carried out using a medium containing no tween 80 with similar results.

These results suggest that under the above conditions the H37RV strain does not become resistant to the tuberculostatic action of *p*-aminosalicylic acid. In an attempt to obtain further and possibly more significant data we obtained cultures of *M. tuberculosis* isolated before and during treatment from patients suffering from pulmonary tuberculosis who received 20 g./day for 6/day week of sodium *p*-aminosalicylate given orally in divided doses. The strains, after cultivation in Dubos medium, were subjected to sensitivity tests by the method described in our previous paper⁴ using a standard inoculum (0.001 mg./ml. of dry bacterial substance). The results of these experiments are shown in Table I and it will be seen that in only one instance (case 9) was there any indication of development of resistance to *p*-aminosalicylic acid. It is of interest to note, however, that there does exist a slight difference in sensitivity between different strains. These results together with those of

PARA-AMINOSALICYLIC ACID. PART IV

Lehmann², Hurni¹, and Seivers³ suggest that "drug-fastness" is not a significant problem in the treatment of tuberculosis with this drug.

Whilst this paper was in course of preparation, Graessle and Pietrowski⁵ reported that repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 120 days failed to produce an increase in the resistance of the strain.

TABLE I

Case No	Before treatment	<i>p</i> -Aminosalicylic Acid treatment		
		2 months	3 months	4 months
1	0 0243—0 0121			0 0975—0 0487
2	0 0487—0 0243	0 0243—0 0121		
3	0 0243—0 0121			0 0487—0 0243
4	0 0243—0 0121			0 0487—0 0243
5	0 0243—0 0121			0 0487—0 0243
6	0 0243—0 0121			0 0243—0 0121
7	0 0487—0 0243	0 0487—0 0243		0 0487—0 0243
8	0 0487—0 0243	0 0487—0 0243		0 0487—0 0243
9	0 0243—0 0121			3 125 —1 56
10	0 0243—0 0121	0 0243—0 0121		0 0121—0 0096
11	0 0487—0 0243	0 0487—0 0243		0 0487—0 0243
12	0 0487—0 0243	0 0243—0 0121		0 0487—0 0243
13	0 39 —0 195	0 39 —0 195		0 0975—0 0487
14	0 0487—0 0243	0 0487—0 0243		0 0487—0 0243
15	0 0243—0 0121	0 0243—0 0121		0 0243—0 0121
16	0 0243—0 0121			0 0243—0 0121
17	0 0487—0 0243		0 0487—0 0243	
18	0 0487—0 0243		0 0487—0 0243	
19	0 0243—0 0121	0 0243—0 0121		
20	0 0243—0 0121	0 0243—0 0121		
21	0 0487—0 0243			0 0487—0 0243
22	0 0243—0 0121			0 0243—0 0121
23	0 0243—0 0121	0 0243—0 0121		
24	0 0243—0 0121	0 0243—0 0121		
25	0 0487—0 0243		0 0487—0 0243	

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SOME ASPECTS OF THE PHARMACOLOGY OF *PARA*-AMINOSALICYLIC ACID

BY E. M. BAVIN

(From the Pharmacological Laboratory, British Chemicals and Biologicals Ltd., Loughborough)

Received July 2, 1949

Para-AMINOSALICYLIC acid has now become of such general interest that it is hardly necessary to quote in detail the many references in the literature to its chemistry, pharmacology and clinical value. Most of the studies on the pharmacology of this substance have been carried out in Sweden^{1,2,3,4,5} and America^{6,7,8,9,10} and little has so far been published in this country. The object of the present series of experiments was to confirm, if possible, certain aspects of the published work and to extend the study of the pharmacology of *p*-amino-salicylic acid in other directions.

The acute and chronic toxicity of the drug in the strain of mice used in this laboratory was first determined, together with the pathological effects of prolonged administration. Blood levels and rate of excretion in mice were studied and following the work of Beyer^{11,12} the possible effect of 4-carboxy-phenylmethanesulphonanilide (Caronamide) in retarding excretion was investigated. Erdei and Snell¹³, Nagley and Logg¹⁴ and other clinical workers have commented on the fall in temperature produced in tuberculous patients treated with *p*-aminosalicylic acid and it seemed desirable to study the possible antipyretic effect of the drug. O'Connor¹⁵, bearing in mind the known action of salicylates, referred to the possibility of causing hypoprothrombinæmia by the use of *p*-aminosalicylic acid, and it was felt that experimental evidence on this point, also, would be useful.

The chemotherapeutic action on *Mycobacterium tuberculosis* in mice was investigated, using a technique very similar to that described by Youmans and McCarter¹⁶. A report by Woody and Avery¹⁷ that potassium iodide potentiated the action of streptomycin in guinea-pigs infected with tubercle suggested that this work should be repeated using *p*-aminosalicylic acid as the tuberculostatic drug.

EXPERIMENTAL METHODS

Toxicity. White Swiss mice of both sexes weighing 20 to 25 g. were used. In acute experiments, the drug was administered intravenously, subcutaneously, intraperitoneally and orally as an aqueous solution of the sodium salt, and observations of mortality were continued for 7 days after injection. Oral doses were given by stomach tube under light ether anaesthesia. In chronic experiments, the drug was given dissolved in the drinking water and the amount consumed daily per cage of 10 animals was measured. The solution was provided in drinking bulbs with constricted outlets, so that the animals were able to obtain liquid by licking the ends of the tubes but no loss of the solution by spilling occurred.

Blood levels. Blood samples were obtained at intervals after administration, by decapitation of the mice. Estimations were carried out by the method described below.

Antipyretic effect. Rabbits of a mixed stock and both sexes, weighing 2 to 3 kg., were used. Pyrexia was induced by intravenous injections of a solution of pyrogen prepared from *Pseudomonas aeruginosa* by the method of Welch *et al.*¹⁸. Rectal temperatures were determined with clinical thermometers. *p*-Aminosalicylic acid and known antipyretics were administered intravenously and orally.

Prothrombin Times. Prothrombin times on rabbits were determined by Quick's method¹⁹. Blood samples were removed from the ear vein by syringe containing 0.1 ml. of 0.10M sodium oxalate per ml. of blood.

Chemotherapy. Mice were used of the same strain as those used for the toxicity experiments. They were infected with *M. tuberculosis* H37 Rv. These organisms were cultured in a modified Dubos medium containing NaH_2PO_4 1.0 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 6.25 g.; Sodium citrate 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6 g.; Casein hydrolysate 2.0 g.; Tween 80 0.5 g.; tap water 1000 ml. Cultures for mouse inoculation were grown in the above medium for 10 days at 37°C. Most of the clear supernatant was poured off and the deposit harvested. A 5 ml. sample of the thick suspension was assayed for moist weight of organism per ml. by centrifuging, resuspending in a small amount of alcohol (50 per cent.) and centrifuging again in a tared tube. The supernatant liquid was poured off and all excess of moisture was removed with a small cotton wool swab. This method appeared to give reasonably reproducible results when several assays were made on one suspension. The assayed suspension was standardised to twice the concentration required finally, using the modified Dubos medium.

Fresh egg yolks were separated and shaken with an equal volume of sterile saline and strained through several layers of muslin. Material for inoculation was prepared by mixing equal parts of egg yolk mixture and double strength culture suspension. 0.25 ml. of this suspension containing 0.3 mg. moist weight of organisms (occasionally 0.1 or 1.0 mg.) was injected intravenously into each mouse.

The survival time of untreated mice infected with 0.3 mg. of organisms was usually between 15 and 30 days and survival time over a period of 28 days was used as the main criterion of the protective action of drugs. Post-mortem examinations were carried out on the heart, lung, liver, spleen, kidney and gut for macroscopic lesions and in certain cases microscopical examinations of fixed sections were made. We have not found the close correlation between post-mortem appearance and dose of drug reported by Raleigh and Youmans.²⁰

The drug under examination was administered either subcutaneously, twice a day, into the interscapular region or orally in the drinking water, as described for the toxicity experiments. In the latter case, measurements were made of the daily consumption per cage of 5 mice. Dosage was commenced within an hour or two of infection, with the exception of the curative experiments where the drug was given 14 days after infection.

ESTIMATION OF *p*-AMINOSALICYLIC ACID

After trials of the numerous published methods, we find the following modification of the method described by Klyne and Newhouse²¹ the most useful for our purpose.

(i) *Determination in Blood.*

Reagents: Solution of *p*-toluenesulphonic acid 20 per cent. in 0.2N hydrochloric acid.

Hydrochloric acid, 10 per cent. v/v.

0.75M disodium hydrogen citrate prepared by mixing 39.4 g. of citric acid with 16.8 g. of sodium hydroxide and diluting to 250 ml.

Solution of *p*-dimethylaminobenzaldehyde 2 per cent. in alcohol (95 per cent.).

1.0 ml. of oxalated blood is added to 13 ml. of distilled water with shaking. After 3 minutes, 6 ml. of *p*-toluenesulphonic acid reagent is added slowly with shaking. The mixture is filtered through a Whatman No. 4 paper and refiltered if necessary to give a clear solution. To 5 ml. of the filtrate, add 1.0 ml. of the citrate buffer, 0.4 ml. of 10 per cent. hydrochloric acid and 1.0 ml. of *p*-dimethylaminobenzaldehyde reagent. Make up to 10 ml. and read on the Spekker absorptiometer after 15 minutes, using 0 B1 filters and a 1 cm. cell. A blank is similarly prepared from normal blood.

(ii) *Determination in Urine.*

Reagents:—Trichloroacetic acid, 10 per cent. w/v.

Solution of sodium hydroxide 1 per cent.

Hydrochloric acid, 10 per cent. v/v.

Ehrlich's reagent (1 g. of *p*-dimethylaminobenzaldehyde in 3.3 ml. of concentrated sulphuric acid diluted to 50 ml. with distilled water).

(a) *Procedure for free p-aminosalicylic acid.*

The urine is diluted to 50 volumes with distilled water and 1 ml. of the diluted urine added to 9.0 ml. of the following mixture:—Trichloroacetic acid 10 per cent. w/v 32 ml.; solution of sodium hydroxide 1 per cent. 30 ml.; Ehrlich's reagent 10 ml.; distilled water 18 ml. The colour is read on the Spekker absorptiometer using 0B1 filters and 1 cm. cells.

(b) *Procedure for total p-aminosalicylic acid.*

To 1.0 ml. of urine 2 ml. of hydrochloric acid 10 per cent. v/v is added and the whole diluted to 10 ml. with distilled water. This diluted urine is heated on a water-bath at 100°C. for 1 hour, when all the acid free, and conjugated, is decarboxylated to *m*-aminophenol. The solution is then diluted to 25 ml. and the *m*-aminophenol estimated by the method described for free acid, substituting the 1 : 25 acid solution for the 1 : 50 aqueous dilution. Standard curves are used to convert the extinction coefficients into *p*-aminosalicylic acid concentrations in all estimations.

PHARMACOLOGY OF PARA-AMINOSALICYLIC ACID

RESULTS

Toxicity. The sodium salt was administered but the doses in Table I are expressed as the acid. Mortality figures give the number of mice dead 7 days after administration.

TABLE I
ACUTE TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Dose in mg./g.	6 0	5 0	4 0	3 5	3 25	3 0	2 5	2 0
Mortality intraperitoneally	5/5		1 5	0 5		0/5		
Mortality intravenously		5 5	2 2	3/3	5/5	5/5	2/5	0.5
Mortality subcutaneously	5/5		2 5			0 5		
Mortality orally		5/5	2 5			1 5		

From these figures, the approximate LD50 for the various routes appear to be as follows:—Intraperitoneal 4.5 mg./g.; Intravenous 2.5 mg./g.; Subcutaneous 4.0 mg./g.; Oral 4.0 mg./g.

(b) *Chronic Experiments.*

TABLE II
CHRONIC TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Concentration in Drinking water	Average Consumption in mg./mouse/day	Limits of Average Consumption mg./mouse/day	Mortality after 12 weeks
per cent			
1 0	53 3	22 0—70 0	7/10
0 75	40 6	27 0—50 0	5/9
0 5	24 6	13 4—40 0	0 10

From these figures, assuming the average weight of a mouse to be 20 g., it would appear that the approximate LD50 for daily administration over a period of 3 months is slightly less than 2.0 mg./g. The maximum tolerated dose under the same conditions would be rather more than 1.25 mg./g.

Subcutaneously, 1.25 mg. and 2.5 mg./mouse given twice daily for 8 weeks caused no deaths in groups of 5 mice. Comparable figures are not readily available in the literature, the majority of workers being content to quote the maximum tolerated intravenous dose to rats and rabbits to be more than 2.0 mg./g. and 0.5 mg./g. respectively. In guinea-pigs, single oral doses of 3 mg./g. produced 30 per cent. mortality. The same authors found that daily oral doses of 0.5 mg./g. to guinea-pigs produced a mortality of 70 per cent. after 32 doses. It would seem, therefore, that mice are rather more resistant than guinea-pigs to prolonged oral administration of *p*-aminosalicylic acid. Other workers, ^{2,7,8} have reported toxicity figures based on concentrations but, in the absence of records of consumption, it is not possible to compare them with those obtained in the present experiments. Levaditi *et al.*²² appear to be the only other workers who have administered

oral doses to mice over a long period (75 days) and their figures of 2.5 mg./g. for a toxic dose and 1.0 to 1.5 mg./g. for a tolerated dose agree well with those given above.

A histological examination of the tissues of the mice used in the chronic series of experiments was made either at the time of death or, if the animals survived the test period, at the end of the test. Many of the kidney sections showed a cloudy swelling with some congestion of the tubules and the appearance of a deposit in the lumen. The glomeruli appeared normal. Many of the liver sections showed a loss of cell outline, with granulation of the cytoplasm and signs of nuclear degeneration. The hearts, lungs and small intestines appeared normal in nearly all cases.

Blood levels. Following oral doses of 0.5 mg./g. to mice, the blood level rose rapidly to about 30 mg./100 ml. in 1 hour and fell quickly to zero after about 4 to 5 hours. This is in agreement with earlier reports using rabbits and guinea-pigs^{2,10}. Caronamide given simultaneously with *p*-aminosalicylic acid in oral doses of 0.125 to 0.5 mg./g. had no effect on the peak blood level but appeared to delay somewhat the fall in blood levels. (Fig. 1.)

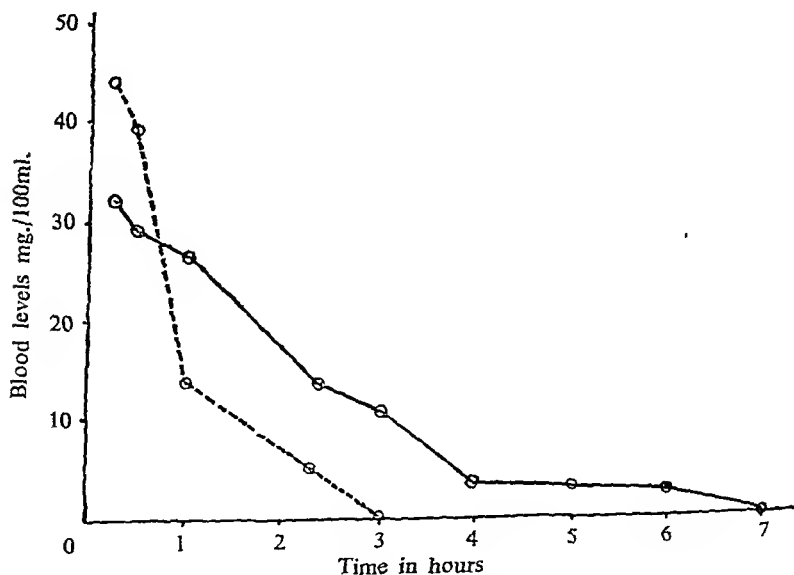


FIG. 1.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in mice

O———O { *p*-Aminosalicylic acid 0.5 mg./g. orally
 O-----O { Caronamide 0.5 mg./g. orally
 p-Aminosalicylic acid 0.5 mg./g. orally

This result was not obtained regularly and, in any case, did not appear to be of the same magnitude as that obtained with penicillin (Fig. 2). However, it seemed worthy of trial in human volunteers and Figure 3. shows the negligible effect exerted by caronamide on the blood levels of *p*-aminosalicylic acid in two normal men.

Apart from the blood concentration produced in mice by one large

PHARMACOLOGY OF PARA-AMINOSALICYLIC ACID

dose, it appeared desirable to examine the blood levels occurring under the conditions of the therapeutic test. Accordingly, determinations were made on mice which had been receiving 0.125 per cent. of the acid in their drinking water (≈ 0.25 mg./g./day) for 4 days. The value, 1.2 mg./100 ml., was almost too low to measure with any degree of accuracy. Nevertheless, this level, as will be seen, is adequate to protect mice for a considerable time against tuberculosis infection, and this fact would seem to suggest that high blood levels may not be essential in clinical treatment, or that the acid is converted *in vivo* into a more active compound.

Antipyretic effect. *p*-Aminosalicylic acid and aspirin were administered orally to rabbits, simultaneously with an intravenous injection of bacterial pyrogen. Rectal temperatures were taken hourly for 5 hours thereafter,

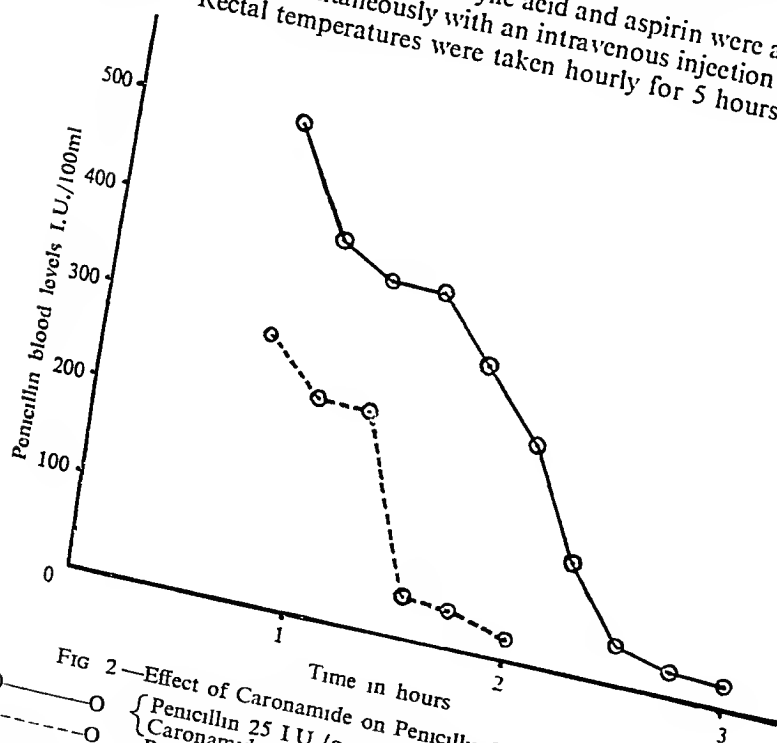


FIG 2—Effect of Caronamide on Penicillin blood levels in mice
 O—O—O { Penicillin 25 I.U./g subcutaneously
 O-----O { Caronamide 0.5 mg./g. orally
 Penicillin 25 I.U./g subcutaneously

and the average maximum rise of temperature calculated. No significant difference was noted between the control group and that receiving the acid, whereas a significant fall in temperature occurred in those animals treated with aspirin. It seems clear that *p*-aminosalicylic acid, under these conditions, has no antipyretic action.

Effect on Prothrombin Times. Two experiments on rabbits were carried out. In experiment 1, normal prothrombin times were determined twice at an interval of 1 week, followed by daily oral doses of 0.5 g. After 3 days' treatment no significant change of prothrombin time had

TABLE III
ANTIPYRETIC EFFECT OF *p*-AMINOSALICYLIC ACID

Number of Rabbits	Dosage	Average maximum rise in temperature °F.
12	Standard Pyrogen 1 : 250 Dilution 5 ml./rabbit intravenously	1.91
11	Standard Pyrogen as above + 1.0 g./kg. of acid orally	1.7
12	Standard Pyrogen as above + 200 mg./kg. of aspirin orally	1.27

occurred. In experiment 2, only one normal level was determined, followed by daily intravenous doses of 0.5 g. After 5 days, a statistically significant increase in prothrombin time was observed, but this fell again to a non-significant level after 12 days. Table IV gives the results of these experiments and it would appear that, on the whole, *p*-aminosalicylic acid, in the dose used, has very little effect on the prothrombin times of rabbits.

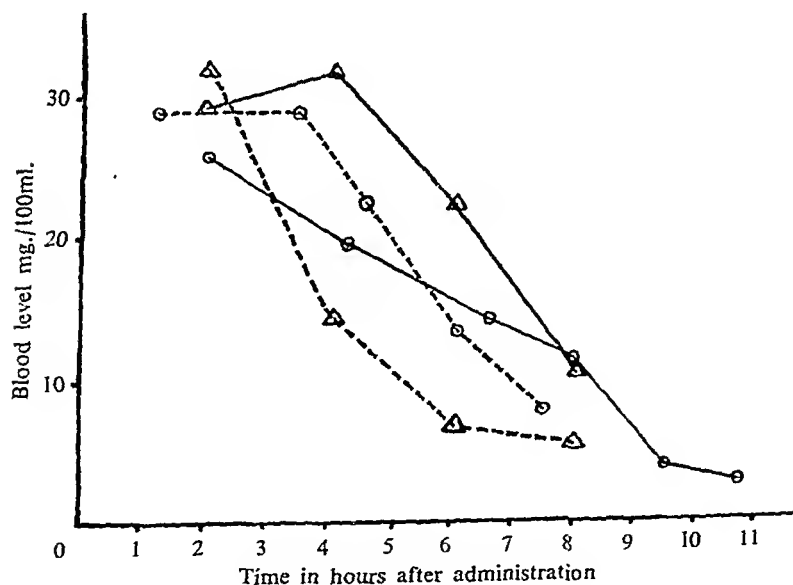


FIG. 3.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in human subjects

- (All doses administered orally)
- Δ———Δ Subject A 20 g. of *p*-aminosalicylic acid
+ 3 g. of caronamide; 4 hours later 3 g. of caronamide.
 - Δ-----Δ Subject A 20 g. of *p*-aminosalicylic acid
 - O———O Subject B 20 g. of *p*-aminosalicylic acid
+ 3 g. of caronamide; 4 hours later 3 g. of caronamide.
 - O-----O Subject B 20 g. of *p*-aminosalicylic acid

PHARMACOLOGY OF *para*-AMINOSALICYLIC ACID

TABLE IV

EFFECT OF *p*-AMINOSALICYLIC ACID ON THE PROTHROMBIN TIMES OF RABBITS

Experiment	Number of Rabbits	Daily Dose	Average Prothrombin times in seconds			
			Normal	After 3 days treatment	After 5 days treatment	After 12 days treatment
1	4	0.5 g. of acid per rabbit orally	10 5 . 9 25	9 95		
2	5	0.5 g. of acid per rabbit intravenously	8 1		10 5	9.5

Therapeutic action. The first experiments were directed towards determining the sensitivity of the test to graded doses of drug and infective organisms. Table IV gives the average survival rates of fairly large numbers of mice under varying conditions, treated with graded oral doses.

TABLE V

EFFECT OF *p*-AMINOSALICYLIC ACID ON SURVIVAL RATES OF MICE INFECTED WITH *M. tuberculosis*

Concentration in drinking water	Acid consumed mg /mouse day	Infecting dose of organism H37 RV (intravenously)		
		1.0 mg	0.3 mg.	0.1 mg
per cent.		per cent	per cent	per cent.
0.25	10.0 approx	68 (15)	90 (20)	90 (20)
0.125	5.0 approx	30 (20)	83 (35)	100 (20)
0.0625	2.5 approx	20 (15)	55 (40)	87 (15)
nil	nil	0 (25)	18 (65)	56 (25)

Figures in brackets indicate the number of mice used.

It will be seen that the test offers a reasonable degree of discrimination, particularly in the group receiving 0.3 mg. of organism. Streptomycin was used as a standard of comparison and Figure IV shows the survival rates of mice infected with 0.3 mg. H37 RV and treated with twice-daily subcutaneous injections of streptomycin or oral doses of *p*-aminosalicylic acid.

Taking the area of each curve as a measure of the comparative action of the respective doses of the two drugs, we found that streptomycin administered subcutaneously was between 3 and 6 times more active than *p*-aminosalicylic acid orally.

TABLE VI

COMPARATIVE THERAPEUTIC ACTION OF *p*-AMINOSALICYLIC ACID AND STREPTOMYCIN SUBCUTANEOUSLY

Daily dose				Mortality after 8 weeks	Average survival times	Average grade of lung lesions
2.5 mg. of acid	0/5	56 days (max.)	3.75
750 µ g. of streptomycin	0/5	56 days (max.)	2.6
375 µ g. of streptomycin	2/5	44 days	4.0
Nil	5/5	18.2 days	4.0

When both drugs were given subcutaneously, the results were as shown in Table VI. Giving some weight to the observations on the lung lesions, it would appear that streptomycin is about 5 times more active than *p*-aminosalicylic acid when both drugs are given subcutaneously.

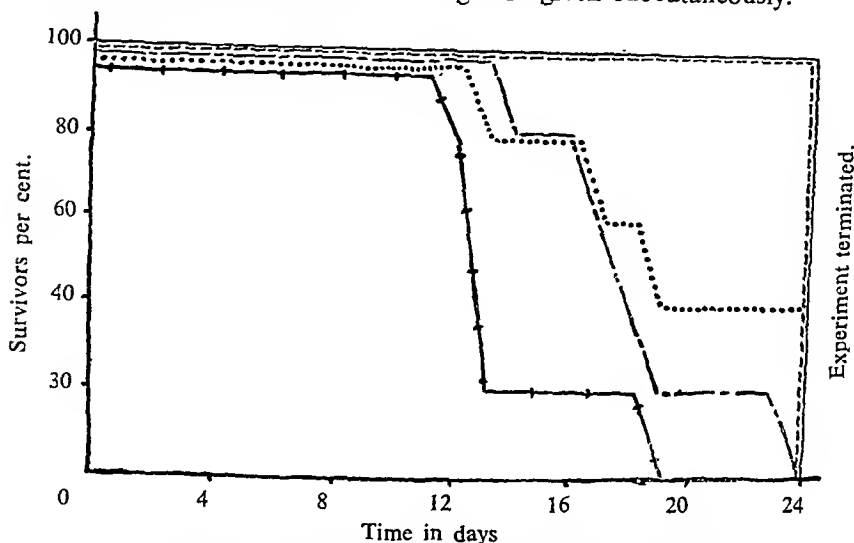


FIG. 4.—Comparison of *p*-Amineosalicylic acid (orally) with Streptomycin (subcutaneously) against experimental tuberculosis in mice

- 2 × 375 µg. of streptomycin daily.
- - - 0.125 per cent. *p*-aminosalicylic acid (c. 5.0 mg./day)
- 2 × 187.5 µg. of streptomycin daily
- + - + - 0.0625 per cent. *p*-aminosalicylic acid (c. 2.5 mg./day)
- + — + — Controls

Curative Action. It was realised that the above type of experiment, although quite useful for screening purposes, did not reproduce the conditions occurring clinically, where an established infection has to be treated. Some experiments were, therefore, carried out using mice in which the infection had been allowed to develop, as judged by the histological examination of control animals, before treatment with *p*-aminosalicylic acid was commenced. Figure V gives the result of such an experiment, from which it will be seen that the acid has some curative effect, although, naturally, it is not so effective as when given from the commencement of the infection. Further experiments on these lines are being carried out.

Development of resistance. Evidence that resistance does not develop either experimentally or clinically has already been reported ^{4,14,23,24,25}. Our own experiments, so far, confirm these reports and it is unnecessary to report them in detail. Briefly, the experiments were of two types, one in which the infective organism (H37 RV) had been grown in medium containing the acid and had then been used to infect mice which were subsequently treated with *p*-aminosalicylic acid, and the second in which the infective organism was obtained from mice which had been treated with *p*-aminosalicylic acid for a considerable period.

PHARMACOLOGY OF *PARA*-AMINOSALICYLIC ACID

In the first case, the organism had been grown in a medium containing 1.0 mg./ml. of *p*-aminosalicylic acid and mice infected with this strain were found to be quite as responsive to the therapeutic action of the acid as mice infected with a similar strain grown in a normal medium.

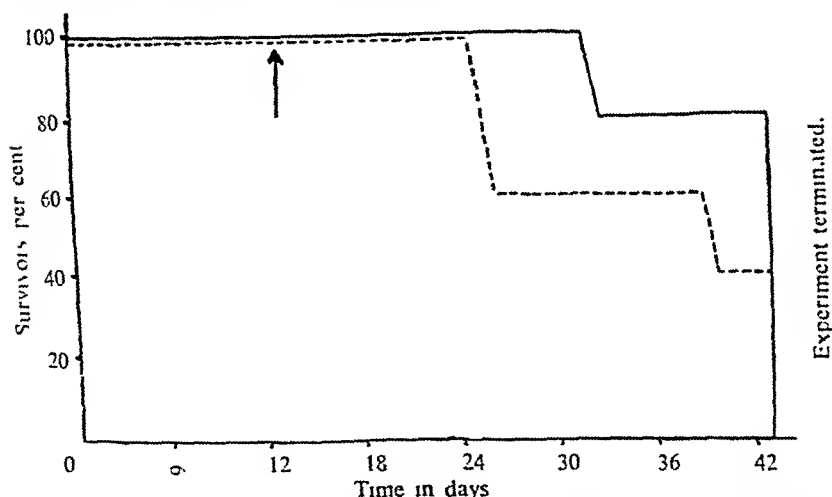


FIG 5—Effect of *p*-aminosalicylic acid against an established tubercular infection in mice

————— 0.125 per cent *p*-aminosalicylic acid (c. 5.0 mg./day)
 - - - - - Controls
 ↑ Dosage of *p*-aminosalicylic acid started

In the second case, the strain of organism was isolated from mice which had received 10 mg./day orally for 42 days and, here again, mice infected with this strain showed no sign of resistance. These results would appear to support previous work that resistance does not occur to any large extent, but, in view of the suggestion²⁶ that it is not easy to demonstrate resistance to streptomycin experimentally in mice, it was thought advisable to repeat the second type of experiment using more passages through *p*-aminosalicylic acid-treated animals and with streptomycin controls. These experiments are still in progress and will be reported at a later date.

TABLE VII
EFFECT OF POTASSIUM IODIDE ON THE TUBERCULOSTATIC ACTION OF
p-AMINOSALICYLIC ACID

Concentration in drinking water	Mortality	Average survival times
0.0625 per cent of acid	7/10	31.3 days
0.0625 per cent of acid — 0.04 per cent of potassium iodide	7/10	39.3 days
0.04 per cent of potassium iodide	8/10	19.0 days
Nil	5/5	18.2 days

Potentiation by Potassium Iodide. Woody and Avery¹⁷ have reported that potassium iodide has a marked potentiating effect on the action of streptomycin in tuberculous guinea-pigs. It was decided to investigate the effect of potassium iodide on the therapeutic action of *p*-aminosalicylic acid, and Table VII gives the results. Statistically, the difference in survival time is not significant. The effect of potassium iodide is not so marked as reported by Woody and Avery for streptomycin, but the above experiment is being repeated using increased concentrations.

DISCUSSION

The experiments in which *p*-aminosalicylic acid was given in the drinking water are of a more quantitative character than those previously described, inasmuch as the daily drug consumption has been measured. This has enabled us to determine a rather more accurate maximum oral tolerated dose over a period of 3 months and to make a comparison of the tuberculostatic action of streptomycin and *p*-aminosalicylic acid. Parenterally or orally, *p*-aminosalicylic acid appears to be much less active than streptomycin parenterally.

Caronamide has been shown to have little or no action on *p*-aminosalicylic acid blood levels and it seems likely therefore that the renal mechanism for the excretion of the acid is different from that obtaining in the case of penicillin. This antibiotic has been shown to be excreted largely *via* the tubules and the experimental result suggests that *p*-aminosalicylic acid may be excreted mainly by the glomeruli.

p-Aminosalicylic acid has been shown to have no effect on the prothrombin times of rabbits, and it would appear therefore that it does not form any derivative of salicylic acid capable of affecting the prothrombin times and that prolonged use is unlikely to lead to hæmorrhagic states.

The contrast between the lack of antipyretic effect shown by *p*-aminosalicylic acid experimentally and the reports of such an effect clinically may be due to the difference in sensitivity of rabbits and man to antipyretic drugs. The dose of acetylsalicylic acid necessary to exert a marked antipyretic effect in rabbits is considerably larger, weight for weight, than for a similar effect in man, and other workers²⁷ have reported similarly large doses of other antipyretics as being required by rabbits. Co Tui and Schrift's report²⁸ of the relative insensitivity of rabbits to pyrogen compared with man is probably another demonstration of this disparity. Brownlee²⁹ has very recently shown that *p*-aminosalicylic acid has a peripheral vaso-dilating action in the human subject, and suggests that the heat loss so produced may explain the drug's antipyretic effect. In a fur bearing animal, such as the rabbit, the vascular effect would be unlikely to produce such a marked heat loss and this may be an alternative explanation of the different action of the drug in rabbits and man. It is proposed to investigate further the antipyretic action of *p*-aminosalicylic acid using partially depilated rabbits.

Resistance to *p*-aminosalicylic acid does not seem to occur, nor does

potentiation by potassium iodide, but further work on both these points is desirable.

SUMMARY

1. Acute and chronic toxic doses of *p*-aminosalicylic acid to mice have been determined by various routes of administration.
2. Prolonged oral and subcutaneous administration to mice produces some pathological effects on the liver and kidney.
3. A method is described for the estimation in blood and urine, and blood levels curves are given in mice and man.
4. Caronamide has no effect on the blood levels in man.
5. It appears to have no hypoprothrombinæmic or antipyretic effect in rabbits.
6. It exerts a protective effect in mice infected with *M. tuberculosis* H37 Rv, but it is not so effective an antitubercular drug as streptomycin.

My grateful thanks are due to my colleagues Mr. A. S. Beach, Mr. J. H. Marvin, Mr. T. R. Middleton and Mr. C. R. B. Williamson for their assistance in this work, and I am indebted to the Directors of British Chemicals and Biologicals Ltd., for permission to publish this paper.

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THE SEPARATION AND IDENTIFICATION OF ERGOT ALKALOIDS BY PAPER PARTITION CHROMATOGRAPHY

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DURING the long history of ergot much time has been devoted to the study of the alkaloids, to which the drug owes its therapeutic and toxic properties. Many workers have contributed to our knowledge of this subject and, in particular, the problem of assaying ergot has been a major topic, engaging the attention of laboratories all over the world. A good summary of analytical work up to 1937 has been given by Barger¹ and a later account has more recently been published by the American Pharmaceutical Association². In spite of the effort already expended on this project, however, available methods still lack specificity; biological assays measure the total potency due to the alkaloids in the preparation under examination, while colorimetric and other chemical methods estimate the total alkaloids, the water-insoluble or the water-soluble alkaloids. The results may be stated in terms of alkaloid calculated as ergotoxine, ergotamine or ergometrine but no method so far available will allow the actual amounts of these alkaloids in a specimen of ergot to be determined.

It was the purpose of the work, described in the present communication, to apply the technique of paper partition chromatography³ to this problem for, in view of the remarkable success of this new technique in other fields, there was good reason to believe that results of interest would emerge.

SEPARATION OF ALKALOIDS

The separation of amino-acids on paper chromatograms using a water saturated solvent, first described by Consden, Gordon and Martin³, is well known. These workers used strips of filter paper, on which were placed spots of the solutions under test, the upper end of the paper being immersed in a horizontal trough containing the water-saturated solvent. The strips were hung in an airtight chamber in an atmosphere saturated with water and solvent. The solvent from the trough gradually passed down the paper causing separation of the amino-acids which were subsequently located on the paper by use of the ninhydrin reagent. A suitable chamber was provided by using a stoneware drain-pipe standing vertically and closed by a lead tray at the bottom and a sheet of plate glass at the top. Water saturated with solvent was placed at the bottom of the chamber in order to maintain the required atmosphere. Full details are given in the original paper, to which the reader is referred.

Using this technique and employing *n*-butyl alcohol-acetic acid-water mixture as solvent we investigated the behaviour of ergot alkaloids on Whatman No. 1 paper. The alkaloids gave little or no colour with the ninhydrin reagent but their positions on the paper were readily detected by their fluorescence in ultra-violet light. It became immediately evident

that the water-insoluble alkaloids, of the ergotoxine and ergotamine groups, passed down the paper with the solvent front. However, separation of the water-insoluble from the water-soluble alkaloids occurred and, what is more important, ergometrine and ergometrinine passed down the paper at different rates and consequently could be separated and identified. The solvent employed was prepared by shaking together *n*-butyl alcohol (4 vols.), glacial acetic acid (1 vol.) and water (5 vols.). After standing the upper layer of *n*-butyl alcohol was placed in the trough and used as the moving phase on the chromatogram, while the lower aqueous acetic acid layer was placed in a dish at the bottom of the drain-pipe. Satisfactory chromatograms resulted when the alkaloids were employed as tartrates, lactates or maleates, but when present as sulphates little movement on the paper took place. A chromatogram was prepared by placing 0.05 ml. of solution containing 5 to 10 μ g. of alkaloid on the paper. After allowing the solvent to run down the paper for 12 to 18 hours the chromatogram was air dried and examined using a suitable source of filtered ultra-violet light. With samples of pure ergometrine and ergometrinine, in solution as maleates, the following R_F values were obtained:—Ergometrine, 0.59; ergometrinine, 0.68.



Left: Ergometrine.

Right: Ergometrinine.

Centre: Mixture of Ergometrine and Ergometrinine.

Fig. 1. Section of chromatogram of ergot alkaloids in ultra-violet light, showing the relative positions of ergometrine and ergometrinine.

Figure 1 shows a typical chromatogram, photographed while exposed to ultra-violet light, and illustrates the application of the method for purposes of identification.

An obvious extension of this work is the development of a method for estimating ergometrine, the most important ergot alkaloid. In this we

have only been partially successful. The fluorescent spots corresponding to the respective alkaloids may be marked on the chromatogram with pencil and subsequently measured in area or cut out and extracted to remove the alkaloids. These methods did not yield satisfactory results, however, for the quantitative removal of the alkaloids from the paper proved surprisingly difficult and the area of the spots could not be used for quantitative work. Our most successful results were obtained by preparing a series of standard spots of ergometrine on a paper chromatogram at the same time as the sample under test was examined, and matching the fluorescence of the respective spots under ultra-violet light. As with most fluorescent methods difficulties caused by quenching of the fluorescence at high concentrations were encountered and it was necessary to use solutions containing less than 0.001 per cent. of ergometrine before the fluorescence approached a linear relationship to concentration. Matching was performed visually and the precision was not high, the experimental error being of the order of ± 20 per cent. As an alternative procedure chromatograms were prepared using serial dilutions of the standard and test solutions until dilutions were reached at which the fluorescence of the alkaloidal spots disappeared. This technique failed owing to the difficulty of determining the end-point, for even at extreme dilutions the fluorescence persisted.

THE ERGOMETRINE CONTENT OF ERGOT

The first attempt to determine chemically the water-soluble alkaloids in ergot was made by Hampshire and Page⁴ and their method has formed the basis of the B.P. 1948 assay process, in which the water-soluble alkaloidal content is expressed in terms of ergometrine and estimated from the difference between estimations of the total and water-insoluble alkaloids. Numerous other researches on the same topic have been published but the only processes of note are those developed by Grove⁵ and by Powell *et al.*⁶ by which the ergometrine is extracted and determined directly by colorimetric assay. These latter processes have been further developed in a collaborative study described by Smith⁷ and have also been included in a report on the assay of ergot issued by the American National Formulary Committee⁸.

We have estimated the ergometrine content of ergot, using our chromatographic technique and, for this purpose, the following process was used. 5 g. of ergot, ground to No. 60 powder, was defatted by extraction with light petroleum and air dried at room temperature. The resulting powder was thoroughly mixed with 0.3 g. of sodium bicarbonate and water was added, drop by drop, with stirring until there was obtained a well damped mass, which was then placed in a percolator (made from a piece of glass tubing 1 inch in diameter) and extracted with peroxide-free ether containing 5 per cent. of alcohol. Extraction of the alkaloids was slow and was best performed by drawing off 10 ml. of percolate at hourly intervals until about 70 ml. had been collected, after which the marc and solvent were allowed to remain in contact overnight before further percolate was withdrawn. Collection of the percolate was then continued, as described above, until another 100 ml. had been withdrawn, when the

ERGOT ALKALOIDS

process was stopped and the marc allowed to remain in contact with the solvent overnight. The extraction was completed in the morning by drawing off portions of percolate at half-hourly intervals until the total volume of extract amounted to 200 to 250 ml. The percolate was collected in an amber glass bottle and the whole process carried out in a dark room. After transferring the ethereal extract to a separating funnel the alkaloids were removed by shaking with 6 quantities, each of 10 ml., of 5 per cent. lactic acid; the acid extracts being collected in a graduated cylinder and the volume adjusted to 100 ml. with distilled water. Portions of this extract were then suitably diluted with 1 per cent. lactic acid until 0.05 ml. placed on a No. 1 Whatman paper strip and developed with *n*-butyl alcohol-acetic acid-water mixture, as described in the first section, gave a fluorescent spot approximately equal in intensity to that obtained with an ergometrine standard containing 0.2 to 0.5 µg. of ergometrine in 0.05 ml. By running a series of standards on the same paper the ergometrine content of the ergot was estimated.

Table I summarises the results obtained on samples of ergot, which were also assayed by the process of the B.P. 1948 and by that of the American National Formulary Committee^a.

It will be seen that the N.F. and chromatographic methods, in most cases, gave results for the ergometrine contents which were in reasonable agreement, but that the B.P. process afforded figures for the water-soluble alkaloids far in excess of the ergometrine present. Accordingly some of the final tartaric acid extract containing the total alkaloids, obtained by the B.P. process, was submitted to chromatographic analysis. Besides alkaloids of the ergotoxine group ergometrine and ergometrinine were detected. In addition a slower moving band was present above the ergometrine and this we were able to identify as being due to lysergic and *iso*-lysergic acids. It thus became clear that the use of boiling ether for extraction resulted in partial hydrolysis of the alkaloids with the production of lysergic acid, which was removed together with the water-soluble alkaloids and was estimated as ergometrine. The B.P. process therefore, did not yield reliable figures for the ergometrine content of the drug.

TABLE I

Sample of Ergot	B.P. 1948 Process		National Formulary Committee Process		Ergometrine determined chromatographically
	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	
	per cent.	per cent.	per cent.	per cent.	per cent.
1	0.22	0.043	0.215	0.023	0.019
2	0.10	0.0125	0.12	0.0086	0.006
3	0.19	0.038	0.195	0.025	0.026
4	0.16	0.040	0.195	0.020	0.016
5	0.20	0.045	0.21	0.022	0.024

Some preliminary experiments with liquid extract of ergot B.P. 1914 and liquid extract of ergot B.P. 1932 showed that the presence of ergometrine in these preparations could readily be confirmed by suitably

diluting with 1 per cent. lactic acid and preparing a chromatogram. Rough assays could be carried out as the colouring matter of the extracts remained almost stationary at the top of the chromatograms. Old extracts contained both ergometrine and lysergic acid.

ERGOMETRINE PREPARATIONS

At the British Pharmaceutical Conference 1948 Foster and Stewart⁹ gave an account of the stability of ergometrine preparations. During the discussion on the paper Eastland¹⁰ questioned the conclusion of the authors that the drop in biological potency of ergometrine maleate injection on storage was due to conversion of ergometrine into ergometrinine, and stated that this was at least partially due to hydrolysis of ergometrine to lysergic acid. Eastland supported his views by experimental data showing that while colorimetric assays of ergometrine injection, filled into ampoules under nitrogen and incubated for some months at 45°C., showed little loss of alkaloid by direct assay, a lower figure for the alkaloidal content was obtained if the injection were rendered alkaline and the alkaloid extracted before being estimated. It was suggested that the difference was due to lysergic acid.

During the past year we have carried out direct and indirect assays on ampoules of injection of ergometrine maleate B.P. 1948 which had been stored at room temperature for periods up to 10 years. For indirect assays the alkaloid was extracted with ether after making alkaline and saturating the solution with sodium chloride as described by the N.F. Committee⁹. Preliminary extraction experiments using solutions of pure ergometrine and ergometrinine maleates, of the same strength as used for the injection, showed that 90 to 95 per cent. recovery of ergometrine and 95 to 100 per cent. recovery of ergometrinine resulted. When applied to ergometrine maleate injection the process gave a recovery of 90 to 95 per cent. of the total alkaloidal content when freshly prepared ampoules were employed, but with 5-year-old ampoules the recovery was only 75 to 80 per cent. Allowing for a 10 per cent. loss during the extraction it would appear that some 15 per cent. of alkaloid in the older ampoules remains to be accounted for. The results of Eastland were therefore confirmed.

In order to study the composition of the injection more closely

TABLE II

Sample of ergometrine maleate injection B.P. 1948	Time of storage at room temperature	Components identified on chromatogram
Freshly made and unsterilised ...	—	Ergometrine
Freshly made and sterilised at 10 lb. pressure of steam for 30 minutes	—	Ergometrine Ergometrinine Traces of lysergic and iso-lysergic acids
Sterilised and stored at room temperature	5 years	Ergometrine Ergometrinine Lysergic and iso-lysergic acids

samples were examined by paper partition chromatography using *n*-butyl alcohol-acetic acid-water mixture as solvent. The results are summarised in Table II.

The general results indicated that on sterilisation of the injection some conversion, estimated to be approximately 20 per cent., of ergometrine to ergometrinine occurs. Very little further conversion appears to take place on storage at room temperature which, however, results in slow hydrolysis of the alkaloids with the formation of lysergic and *iso*-lysergic acids. The presence of the lysergic acids is better shown by using a basic solvent prepared by shaking a mixture of *n*-butyl alcohol (4 vols.), water (5 vols.) and pyridine A.R. (1 vol.), allowing to separate and using the *n*-butyl alcohol layer as the moving phase on the chromatogram, while the aqueous layer is used for saturating the atmosphere of the chamber. Under these conditions and using No. 1 Whatman paper the R_f values were as follows:—Lysergic acid, 0.2; *iso*-lysergic acid, 0.4.

The presence of lysergic acid was further confirmed by extracting the alkaloid from some old injection, the final alkaloidal extract being made with 1 per cent. lactic acid. On preparing a chromatogram with this extract ergometrine and ergometrinine were identified but the lysergic acids, present in the chromatogram of the original injection, had disappeared.

It was of interest to examine chromatographically tablets of ergometrine maleate which had been stored at room temperature and, for this purpose, the tablets were extracted with, or dissolved in, 1 per cent. lactic acid. Very little, if any, formation of ergometrinine or lysergic acid was detected in tablets which had been stored for periods up to 5 years.

IDENTIFICATION OF WATER-INSOLUBLE ALKALOIDS

The water-insoluble alkaloids of ergot are distinguished from ergometrine by the presence in their molecular structures of certain amino-acids. Jacobs and Craig¹¹ found among the products of alkaline hydrolysis of ergotinine the lactam of a dipeptide, derived from L-phenylalanine and D-proline, while Smith and Timmis¹² showed that ergosine gave a similar lactam of the dipeptide of L-leucine and D-proline. Stoll, Hofmann and Becker¹³ showed that ergocornine, ergocristine and ergokryptine, isolated from the ergotoxine group of alkaloids, also contained amino-acids of the L-series in addition to D-proline.

TABLE III

ALKALOIDS CHARACTERISED BY STRUCTURES DERIVED FROM LYSERGIC OR *iso*-LYSERGIC ACID, AMMONIA, A KETO ACID, D-PROLINE AND ONE OTHER AMINO-ACID

Additional amino acid	Ergotamine group (pyruvic acid group)	Ergotoxine group (Dimethyl-pyruvic acid group)
L-phenylalanine	Ergotamine Ergotaminine $C_{21}H_{21}O_4N_2$	Ergocristine Ergocristinine $C_{21}H_{21}O_4N_2$
L-leucine	Ergosine Ergosinine $C_{21}H_{21}O_4N_2$	Ergokryptine Ergokryptinine $C_{21}H_{21}O_4N_2$
L-valine	—	Ergocornine Ergocorninine $C_{21}H_{21}O_4N_2$

diluting with 1 per cent. lactic acid and preparing a chromatogram. Rough assays could be carried out as the colouring matter of the extracts remained almost stationary at the top of the chromatograms. Old extracts contained both ergometrine and lysergic acid.

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Sterilised and stored at room temperature	5 years	Ergometrine Ergometrinine Lysergic and iso-lysergic acids

lysis of ergine, the amide of lysergic acid, gave a continuous streak showing a variety of colours, but no major spots reacting with ninhydrin. A similar streak often appeared on chromatograms prepared with hydrolysates of water-insoluble alkaloids but was fainter and caused no difficulty in the identification of the amino-acids.

Using the above technique the results shown in Table IV were obtained with samples of "pure" alkaloids.

From these preliminary experiments it is obvious that not only is paper chromatography a valuable tool for identifying but also for assessing the purity of ergot alkaloids. For example, it is clear that in the cases of the ergocristine and ergocornine examined each alkaloid was contaminated with traces of the other. When assessing the degree of contamination it is necessary to remember that phenylalanine, under the conditions employed, gives less colour than leucine or valine and one of the quantitative applications of paper chromatography should be applied.

Other minor ninhydrin spots have been noted in the chromatograms, but at present no assignment of these to any known constituent of the hydrolysate can be made. Alkaline hydrolysates have not shown the presence of amino-acids but, in general, give two elongated spots reacting with ninhydrin. The substances in these spots have yet to be identified.

DISCUSSION

It was stated early in this paper that available methods of ergot assay lacked specificity and it is felt that the application of paper partition chromatography, now described, has done something to remedy this deficiency.

The need for a specific identification test for ergometrine has long been felt by workers in this field and it is a matter of great importance to the chemical manufacturer who wishes to purchase ergot for the manufacture of ergometrine. By paper chromatography it is a simple matter, even with a small sample of drug, to state whether ergometrine is present and approximately in what quantity. The application to liquid extracts of ergot has also been described.

Of all ergot preparations perhaps injection of ergometrine maleate is the most important. As a result of the present work a much clearer picture of the changes which occur during the manufacture and storage of the injection has been obtained and, in view of the very small quantity of injection required for a test, the chromatographic technique has opened the field for much fuller investigation. It has been seen that conversion of ergometrine to ergometrinine occurs on heat sterilisation of the injection and it may be that sterilisation at room temperature by candle filtration might afford a better preparation. The pH of the injection may influence the alkaloidal equilibrium and we have experiments in progress to explore this aspect of the problem. Hydrolysis of the alkaloid in the injection has been confirmed and this factor must be added to the previously accepted causes of deterioration⁹. It is considered unlikely that the hydrolysis of ergometrine in solution can be avoided.

The close relationship of the ergot alkaloids to the polypeptides has been emphasised by the use of the method of chromatograms, prepared from the

The relationships of the known water-insoluble alkaloids are summarised in Table III embodying data by Stoll *et al*¹³.

The exact mode of linkage of the amino-acids in these alkaloids is not known but Stoll and Hofmann¹⁴ have reported that thermal degradation of ergotamine yielded pyruvyl-phenylalanyl-proline, and Stoll¹⁵ has further reported finding dimethylpyruvyl-valyl-proline, together with lysergic acid amide, in the products from the alkaline hydrolysis of ergocornine.

It is thus clear that the identification of the amino-acids obtained on acid hydrolysis would be of great assistance in the identification of pure alkaloids and, in the absence of major quantities of extraneous amino-acids, might be of help in the examination of cruder preparations. The occurrence of valine in ergocornine and ergocorninine is a specific test for this inter-convertible pair of alkaloids. The presence of phenylalanine would indicate ergotamine or ergocristine and their isomers, further tests such as the identification of the keto-acid and the determination of physical properties being necessary for complete identification of the alkaloids. The finding of leucine would be equally significant for the detection of ergosine and ergokryptine and their isomers.

We have identified the amino-acids in the acid hydrolysates of the alkaloids by the method of paper partition chromatography in the following manner. 10 mg. of the alkaloidal preparation together with 1 to 2 ml. of concentrated hydrochloric acid was heated in a sealed tube at 100°C. for 16 hours. After cooling, the contents of the tube were transferred to an open dish and evaporated to dryness on a steam bath. The dark residue was extracted with 0.2 ml. of distilled water and, without separating the insoluble matter 0.01 ml. of the suspension was placed on the paper. The chromatogram was prepared on Whatman No. 4 paper using *n*-butyl alcohol-acetic acid-water mixture. Although the positions of the amino-acids on the well-dried paper were revealed by their bluish-white fluorescence in filtered ultra-violet light, a more specific test in the presence of fluorescent alkaloids was afforded by the ninhydrin reaction, carried out by spraying the paper with 0.1 per cent. ninhydrin in equal parts of *n*-butyl alcohol and chloroform, drying and then developing by heating in an oven at 100°C. The amino-acids were identified by their R_F values and their ninhydrin colour reactions, leucine and valine yielding reddish purple spots while those due to phenylalanine and proline were grey-blue and yellow respectively. Under the above conditions hydro-

TABLE IV

Alkaloid	Amino-acids, identified chromatographically
Ergotamine	Proline, phenylalanine
Ergotaminine.	Proline, phenylalanine
Ergocristine	Proline, phenylalanine and faint trace of valine
Ergocristinine (ergotinnine) .. .	Proline, phenylalanine and faint trace of valine
Ergocornine (ergotoxine) . . .	Proline, valine and trace of phenylalanine

DISCUSSION

The paper was read by Dr. G. E. Foster.

The CHAIRMAN said that he would particularly like to congratulate the authors on the ingenious method of hydrolysing the alkaloids and separating the elements chromatographically. At last year's Conference he had suggested that the colour test was not really an indication of the amount of deterioration. The work reported in this paper confirmed that and gave valuable new methods of determining the amount of deterioration. He hoped some agreement would be reached on the names for ergot alkaloids.

DR. F. HARTLEY (London) said that preliminary experiments carried out by his colleagues with a similar object to that of Dr. Foster had shown interesting results but they had not been able to pursue them to the extent that Dr. Foster had done. In examining different solvents for the separation of ergot alkaloids on a chromatogram, they had found that *isobutyric* acid gave R_F 0.90. *n*-Butanol-acetic acid gave an R_F value of 0.55 which agreed well with the figure 0.59 given by Dr. Foster.

In addition to the amino acids obtained by the hydrolysis of ergometrine, 2-amino-propanol was produced. This was readily distinguishable from the amino acids by the ninhydrin reaction and in their hands, using *n*-butanol-acetic acid it had an R_F value of 0.33. This observation might assist in studying the deterioration of ergometrine injection and perhaps Dr. Foster could determine the 2-amino-propanol in his five years old sample.

PROFESSOR BRINDLE (Manchester) said that they had been trying chromatography for the estimation of ergot alkaloids and had found difficulty in extracting the alkaloids from the chromatogram. They had tried to extract the ergotoxine and ergometrine (quantitatively) by prolonged extraction in a continuous extractor with ether, but on examination of the chromatograms in ultra-violet light, a fluorescence persisted. They had had the same difficulty in extracting the alkaloids from silica gel. They had achieved more success using kieselguhr and a citrate-phosphate buffer at pH5. The ergotoxine had been recovered quantitatively, but the ergometrine was not so easy. In a deteriorated solution of ergotoxine they had found a difference between the colorimetric assay, done directly on the solution, and that performed on the extracted alkaloids. They were satisfied it was due to lysergic acid, which did not affect the colour test if the alkaloids were extracted first. He had noticed that deterioration varied according to pH. With a solution at pH3 there was good agreement between the colorimetric and biological assays, with the biological result a little below the colorimetric. If deterioration occurred at pH5 or over there was a big difference between the two assays, with the biological result about half that of the colorimetric assay. Had Dr. Foster any observations to make on the considerable difference in the type of deterioration according to pH?

DR. W. MITCHELL (London) asked if the colours of the ultra-violet fluorescence were distinctive for ergometrine and ergometrinine. Dr. Foster had said that the accuracy of the method of comparing the intensity of

hydrolysates of the alkaloids. In spite of the amount of work so far done on the ergotoxine group of alkaloids it seems doubtful whether complete separation of the individual alkaloids has yet been achieved and, in this connection, it is certain that the chromatograms of the amino-acids will be of great value in testing highly purified specimens for traces of other alkaloids. A long-standing controversy as to whether ergotamine and ergotoxine ever occur together in the same ergot¹⁶ has not been resolved and chromatography may well have something to contribute in this field. Preliminary results with chromatograms, prepared from hydrolysates of single sclerotium of *Claviceps purpurea* have indicated that the amino-acids found are a good indication of the alkaloids present in the drug, about which there is still much to be learnt.

SUMMARY

1. A study has been made of the application of paper partition chromatography in the ergot field.

2. A technique for the separation and identification of ergometrine and ergometrinine, when present in mixtures of total ergot alkaloids, has been described.

3. The method has been extended so that approximately quantitative results may be obtained and, in this way, the ergometrine contents of samples of ergot have been estimated.

4. The changes which take place in injection of ergometrine maleate B.P. 1948, during manufacture and storage have been studied chromatographically. It was found that during heat sterilisation some conversion of ergometrine to ergometrinine occurs. On storage some hydrolysis giving rise to lysergic and *iso*-lysergic acids takes place.

5. By preparing chromatograms from the hydrolysates of water-insoluble ergot alkaloids, identification tests for individual alkaloids have been developed.

We wish to thank Mr. H. M. Hood, B.Sc., for the photograph of a chromatogram, Mr. R. L. Grant, M.Sc., for a sample of ergometrinine and Dr. S. Smith for specimens of lysergic and *iso*-lysergic acids. We are also indebted to the Directors of The Wellcome Foundation for permission to publish this paper.

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THE DETERMINATION OF BENZENE HEXACHLORIDE (HEXACHLOROCYCLOHEXANE) IN PHARMACEUTICAL PREPARATIONS

BY W. H. C. SHAW

*From Pharmaceutical Research and Service Laboratories,
Imperial Chemical Industries, Ltd., Blackley, Manchester, 9*

Received July 1, 1949

In addition to its use as an industrial insecticide benzene hexachloride (Gammexane) has proved of value as an external parasiticide in human and veterinary medicine. For this purpose, the biologically active γ -isomer (Lorexane) has been isolated and formulated to yield a variety of pharmaceutical products. The object of the work described here was to provide a simple method for the determination of benzene hexachloride in the presence of the excipients likely to be found in such formulations. Earlier analytical studies of benzene hexachloride have been concerned chiefly with mixed isomers and have described the determination of either (a) γ -isomer in presence of others, or (b) total benzene hexachlorides. Of these, (a) usually involve biological or physico-chemical methods, while (b) nearly all depend on the alkaline dehydrochlorination to trichlorobenzene originally reported by Van der Linden¹.



Several workers have shown that this reaction is quantitative for all isomers when benzene hexachloride is refluxed with alcoholic potassium hydroxide, and that the determination may be completed by either acid or silver nitrate titration of the resultant solution. Goldenson and Sass² have studied the effect of replacing potassium hydroxide by other alkalis, and Howard³ has drawn attention to the possible use of monoethanolamine, which has the advantages of being easily freed from chloride impurity and of not reacting with vegetable oils.

Since the quality of the active agent is capable of independent analytical control, it was considered sufficient that the method should determine total benzene hexachloride, i.e., there was no need for it to be specific for γ -isomer. The main criterion was that it should be effective in the presence of relatively large amounts of excipients and diluents, since the biological potency of the γ -isomer is such that it is rarely used at concentrations above 1 per cent. In view of the simplicity of the reaction, it was decided to investigate more fully alkaline dehydrochlorination as a basis for a general method.

METHODS OF DEHYDROCHLORINATION:

(1) *By Alcoholic Potassium Hydroxide:* The limits of time, temperature and concentration to ensure a quantitative reaction were investigated as follows. A known weight of pure γ -isomer (about 0.4 g.) was reacted with an excess of alcoholic potassium hydroxide at controlled time and temperature, and the ionisable chlorine produced was titrated with silver nitrate. The results obtained are recorded in Table I, and

fluorescence was about ± 20 per cent. That was not a very high accuracy. Was it also necessary to make allowance for the size of the spot? If the standard spot was smaller than the test spot, presumably it would have some effect on the intensity. Was there any possibility of alteration of the ergometrine due to the somewhat prolonged exposure to air? This method for the testing of ergot would be useful, but the limiting factor was the time involved since it appeared to require at least five working days.

DR. R. E. STUCKEY (London) said that in his laboratory they had had some experience in removing amino-acids from chromatograms for their quantitative estimation. Was Dr. Foster sure that the residual fluorescence was due to traces of ergometrine remaining in the paper and not to peptisation of the paper fibres which was liable to occur? He would like to suggest that the chromatogram be run not as a spot but as a band with ten or more spots. This would increase the amount of ergometrine making a chemical or spectrophotometric estimation possible and the amount of ergometrine left in the paper might then be low in comparison with that extracted. Was any information available on that point?

DR. G. E. FOSTER (Dartford) said he was very interested to learn about Dr. Hartley's experience with other solvents. In view of Dr. Stuckey's suggestion they might have to look into the problem of extraction more closely, but so far they had found it impossible to remove the fluorescence completely. As to the difference of pH, they had prepared an ergometrine maleate injection, and adjusted the pH to various values from 3 to 6 by the addition of maleic acid. The chromatograms were obtained after sterilisation, all the spots being put on one paper. As the pH decreased, the amount of ergometrine gradually increased until at pH 3 or 3.5 there had been very little ergometrinine there at all, and the amount of lysergic acid seemed to decrease at the same time.

A recent sample of ergometrine maleate injection from the U.S. had been found to contain very little ergometrinine, but the acidity had been much higher than that of the B.P. injection.

The colour of the fluorescence was the same for ergometrine and ergometrinine. The 20 per cent. error of visual comparison of the fluorescence was within the limits usual in this kind of work. The size of the spots did not seem to be important. Four of the five days required for the method were taken up with percolation. The ratio of total alkaloids to ergometrine was more or less constant in Spanish or Portuguese ergot, and by doing a rough chromatograph extraction it was possible to obtain a quick qualitative result overnight.

Conversion of the alkaloids on the paper did not appear to occur. They had no information on peptisation of the paper fibres. Neither had they done any tests using a band technique.

PROFESSOR H. BRINDLE (Manchester), in answer to Dr. R. E. Stuckey said they had tried increasing the amount of the alkaloids used in paper chromatography. They could not get a quantitative recovery from the band. As they increased the band, the amount of paper was increased, and it was adsorption on the paper which caused the difficulty.

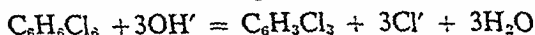
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show that : (a) Using 1 per cent. potassium hydroxide the reaction is quantitative in 15 minutes at room temperature, provided that sufficient alcohol is present to dissolve the benzene hexachloride. (b) No further reaction involving the trichlorobenzenes takes place after at least 1 hour's refluxing with 4 per cent. potassium hydroxide in alcohol (95 per cent.).

TABLE I

	Reaction time	Alcohol strength	Potassium hydroxide solution	Recovery (percentage of theory)
	minutes	per cent. v/v	per cent. w/v	
Room temperature 20° C. approx.	1	95	4	66
	2			89
	5			100.0
	15			100.2
	15		1	99.4
Refluxing	15	Nil (aqueous)	1	16
	15		1	51
	15		1	100.1
	15		1	100.1
	60		4	100.0

(2) *By Monoethanolamine*: Howard³ has already shown that monoethanolamine produces quantitative dehydrochlorination when heated either with solid benzene hexachloride or its oily solutions. To compare this method with (1) the action of an excess of monoethanolamine when used both alone and in alcoholic solution was investigated under similar conditions of time, temperature, and concentration. The results are recorded in Table II and show that:—

(a) *For the alcoholic solution*: (i) Even a large excess of monoethanolamine produced practically no reaction after 1 hour at room temperature. (ii) To obtain a quantitative reaction it was necessary to boil for 2 hours with 8 per cent. of monoethanolamine in alcohol (95 per cent.).

(b) *For monoethanolamine alone*: (i) The reaction was not complete in 18 hours at room temperature. (ii) The reaction was quantitative after 5 minutes heating on the water-bath, with frequent shaking.

The action of monoethanolamine in alcoholic solution is thus much

TABLE II

	Reaction time	Monoethanolamine alone (ml.)	Monoethanolamine (ml. in 25 ml. of alcohol)	Recovery (percentage of theory)
Room temperature	1 hour	—	1	Trace
	18 hours	—	1	16
	18 hours	2	—	91
Refluxing	15 minutes	—	1	24
	60 "	—	1	75
	120 "	—	1	94
	60 "	—	2	97.5
	120 "	—	2	100.0
	5 minutes	2	—	100.0
	15 "	2	—	99.7

DETERMINATION OF BENZENE HEXACHLORIDE

slower than that of potassium hydroxide. This confirms the findings of Howard, who recommended heating with undiluted monoethanolamine for 1 hour at 100°C. as a general procedure for dehydrochlorination.

APPLICATION TO PHARMACEUTICAL PREPARATIONS:

The relative merits of the potash and monoethanolamine methods were assessed for the following types of pharmaceutical preparations, selected as being the most commonly encountered. (a) Solutions in mineral oil (including ointments with paraffin base); (b) solutions in vegetable oil (including sulphonated oils); (c) alcoholic preparations; (d) dusting powders; (e) emulsions and creams. In testing the suitability of each method the following processes, described here as General Methods, were first applied and subsequently modified, where necessary, to suit special requirements.

General Method A (Potassium Hydroxide): To an appropriate weight of sample is added 25 ml. of alcohol, with sufficient potassium hydroxide to react with the benzene hexachloride and with any saponifiable matter present. The mixture is refluxed for 1 hour, cooled, and acidified with dilute nitric acid. Fatty acids, trichlorobenzene, and unsaponifiable matter are extracted with ether and the ionisable chlorine in the aqueous layer determined by any convenient method. A control titration is made to compensate for the presence of ionisable chlorine in sample and reagents. For this purpose, dilute nitric acid is first added in sufficient quantity to ensure that the solution remains acid during and after the addition of potassium hydroxide. It has already been established that benzene hexachloride itself is unaffected by nitric acid⁴.

General Method B (Monoethanolamine): To an appropriate weight of sample is added an excess of monoethanolamine (1 ml. per g. of benzene hexachloride) with 0.1 ml. extra per 1 ml. of oil, and the mixture heated for 1 hour on the water-bath, with frequent shaking. After cooling, the mixture is acidified with dilute nitric acid, diluted with water, and extracted with ether. The ionisable chlorine in the aqueous layer is then determined as before. A blank titration is also carried out as described in Method A. Experimental results are summarised in Table III.

DISCUSSION

The potassium hydroxide method is, in general, the more attractive since the reagent is readily available and the reaction proceeds rapidly in alcohol even at room temperature. It is particularly useful when the analysis involves a break-down of emulsions where monoethanolamine is markedly inefficient. The presence of a trace of chloride impurity in potassium hydroxide of A.R. quality is not a serious disadvantage, firstly because it is small in relation to the total ionisable chlorine produced in the assay and, secondly, because it is compensated by a control titration. This method is thus the more suitable for preparations of types (a), (c), (d) and (e). The only circumstances in which the monoethanolamine method is to be preferred are when it is desired to avoid

TABLE III

Preparation	Prepared Strength per cent w/w	Modifications to Method A	Strength Found per cent w/w	Modifications to Method B	Strength Found per cent w/w
1 Solution in liquid paraffin	1 970	Nil	1 97	Nil	1 96
2 Ointment containing liquid, hard and soft paraffins and wool fat	1 964	Nil	1 96	Not suitable due to formation of emulsions on extraction	—
3 Solution in arachis oil	1 027	Nil	1 01	Nil	—
4 Solution in sulphonated castor oil	5 058	Reflux with nitric acid for control titration	5 09	As for Method A	1 02
5 Solution in aqueous alcohol	0 1013	Add 50 per cent aqueous solution of potassium hydroxide to give a concentration of 1 per cent and allow to stand for 15 minutes. No heating necessary	0 101	Add monoethanolamine to give a concentration of 8 per cent. Reflux 2 hours. Cool, acidify, and complete by General Method	5 06
6 Self emulsifying concentrate containing castor oil and polyglyceryl ricinoleate in alcohol	0 2014	Reflux with dilute nitric acid for control titration	0 202	As for No 5	0 101
7 Dusting powder containing talc and starch	0 102	Extract by shaking with a known volume of cold alcohol (95 per cent). Filter or centrifuge. To an aliquot add sufficient 50 per cent aqueous potassium hydroxide to give a concentration of 1 per cent. Allow to stand 15 minutes, acidify, and complete	0 105	Less suitable than potassium hydroxide due to long procedure	—
8 Ointment with oil in water emulsion base containing sulphonated castor oil, diethylene glycol distearate, and emulsifying wax	0 111	Reflux with dilute nitric acid for control titration	0 107	10 per cent of added monoethanolamine at 100° C for 2 hours failed to give quantitative recovery	—
9 Ointment with oil in water emulsion base containing castor oil, cetyl alcohol, diethylene glycol distearate, polyglyceryl ricinoleate and non-ionic emulsifying agent	0 100	After saponification extract oils, etc with petroleum ether in presence of a high concentration of alcohol	0 103	Comments as for No 8	—
10 Fluid oil in water emulsion containing kerosene and sulphonated castor oil	0 211	Reflux with dilute nitric acid for control titration	0 214	Comments as for No 8	—

DETERMINATION OF BENZENE HEXACHLORIDE

saponification of oils and fats, where the comparatively large quantities of potash required for saponification may introduce an undesirably large amount of chloride. Otherwise, monoethanolamine has the disadvantage of always requiring heat and agitation unless used in high concentration in the presence of alcohol, which eliminates the necessity for shaking. It also becomes less efficient as a dehydrochlorinating agent in the presence of solvents. It is thus recommended, as far as pharmaceutical preparations are concerned, only for the determination of benzene hexachloride in simple solutions in vegetable oils, particularly when the concentration is very small.

SUMMARY

Methods are suggested for the determination of benzene hexachloride in certain pharmaceutical preparations. These are based on alkaline dehydrochlorination using monoethanolamine or alcoholic potassium hydroxide.

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DISCUSSION

DR. C. GARRATT (Nottingham) suggested that the author had obtained a surprising degree of accuracy in the analysis of the difficult mixtures mentioned in Table III. He asked whether the author could give some idea of the degree of variation obtained in successive determinations, and whether the results recorded were typical, or picked from a series which showed the method to advantage.

MR. SEYMOUR (Welwyn) asked whether the author had considered the use of bases other than ethanolamine.

MR. W. H. C. SHAW, replying, said that a certain amount of variation in the analytical results was to be expected. This really depended on the strength of the preparation which was being assayed. With the stronger preparations, the results came in general within ± 0.5 per cent. of the actual amount of benzene hexachloride used, but for the less concentrated preparations such as a 0.1 per cent. cream or emulsion the accuracy probably fell to about ± 2 per cent. As to bases other than monoethanolamine, pyridine had been tried by Howard in the work which led him to recommend monoethanolamine.

AN EXAMINATION OF PYROGEN FROM VARIOUS SOURCES

BY D. W. WYLIE AND J. P. TODD

From the Pharmacy Department of the Royal Technical College, Glasgow.

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WHETHER bacteria in general produce the same pyretic substance or each bacterial species a specific pyrogen is not yet known. During our investigation of pyrogen we have discovered several variations, such as different types of fever curves following injection of modifications of the same culture and variations in stability of pyrogen, from different bacterial sources, when heated at 120°C., which suggest that different pyrogens exist.

FEVER CURVES

A study of published fever curves produced as the result of injection of pyrogen into rabbits^{1,2,3,4,5,6} shows that these can be placed in one or other of three main classes:—1. Curves in which the body temperature started to rise shortly after the injection, reached a peak and then returned to normal again. 2. Curves in which the body temperature started to rise shortly after the injection as in (1), fell slightly, but instead of returning to normal, rose to a second peak, which was then followed by a return to normal. 3. Curves in which there seemed to be a delay before the body temperature rose to its peak, this again being followed by a return to normal. In some of the fevers of this latter type the temperature fell distinctly during the initial period of no reaction, in a few cases this fall being so great that the animals collapsed and sometimes died^{1,6}.

None of the workers in this field appear to have observed any significant difference between the single- and double-peak curves which appeared in graphical form in their publications. This may have been due to the same culture, under what appeared to be exactly the same conditions, stimulating both the single- and double-peak types of curves. It has been shown here that this failure to observe significant differences in the shapes of the curves was probably caused by taking the temperature of the rabbits at hourly intervals only.

In the course of this work, the three types of fevers already discussed were frequently obtained. As the work proceeded, it became apparent that the differences in the curves were significant and were not caused by biological variation or by the method of temperature determination used, but by actual differences in the constitution of the solutions. In experiments in which a fall in body temperature occurred after injection of solutions, the cause was found to be associated not only with the delayed fever (type 3), as suggested in the literature, but to be due to a depressant substance produced by some organisms. After removal of this depressant substance, which can be driven off by gentle heat, the solutions stimulated the single- and double-peak types of fevers.

Typical examples of the three types of fevers are shown in Figure 1. In the single-peak curve (type 1) in which the rise in temperature is immediate, the peak temperature is reached in 70 to 120 minutes, the

PYROGEN FROM VARIOUS SOURCES

average time being 86 minutes; in the double-peak curve (type 2) the first peak is identical with curve type 1, but this falls slightly, to be followed by a second rise, reaching its peak in 3 to 4 hours after the

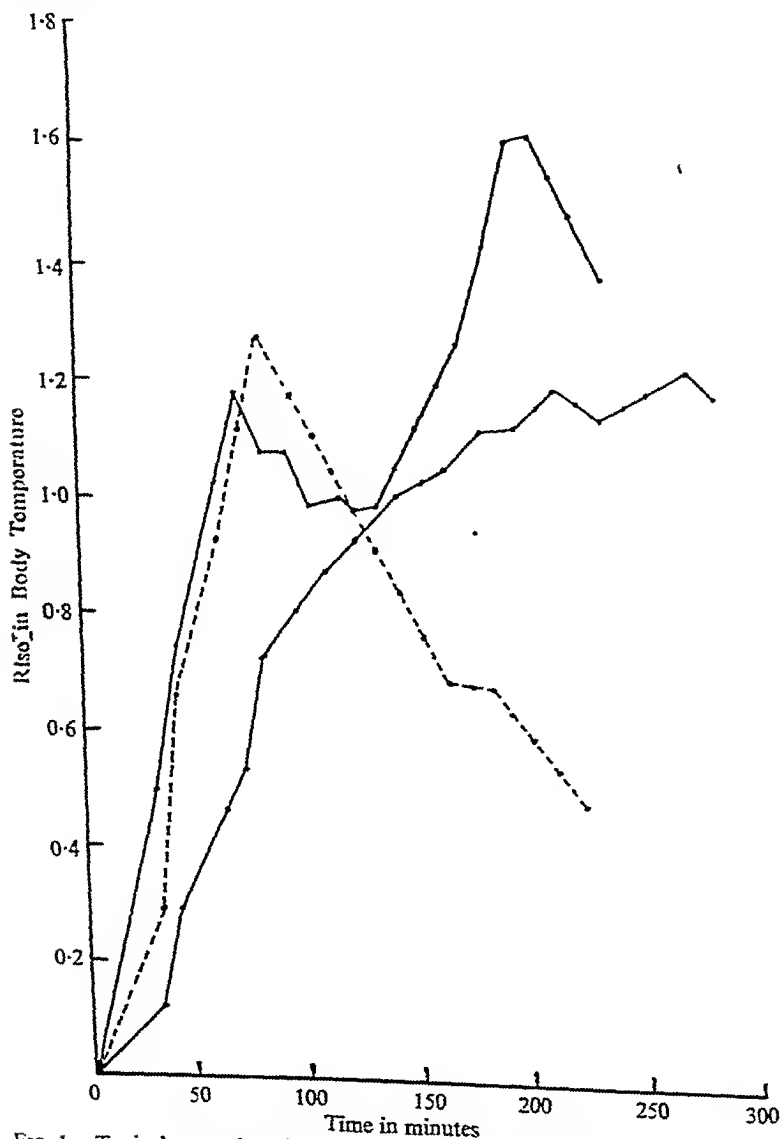


FIG. 1.—Typical examples of three types of fevers stimulated by pyrogenic cultures. injection; in the delayed-peak fever curve (type 3), the time to reach the peak temperature is similar to that of the second peak in the double-peak curve. From a study of the time to reach the peak, it would seem that the double peak curve (type 2) is a combination of the two single-peak curves.

Figure 2 demonstrates how completely the shape of the curve is masked

by determining the rectal temperatures at hourly intervals only, the technique used by most of the investigators, and it is probably due to this that no significant conclusions were drawn from the shapes of the curves, as, when using this method, slight changes in the time to reach the peak could change what appeared to be a single-peak curve in one test to a

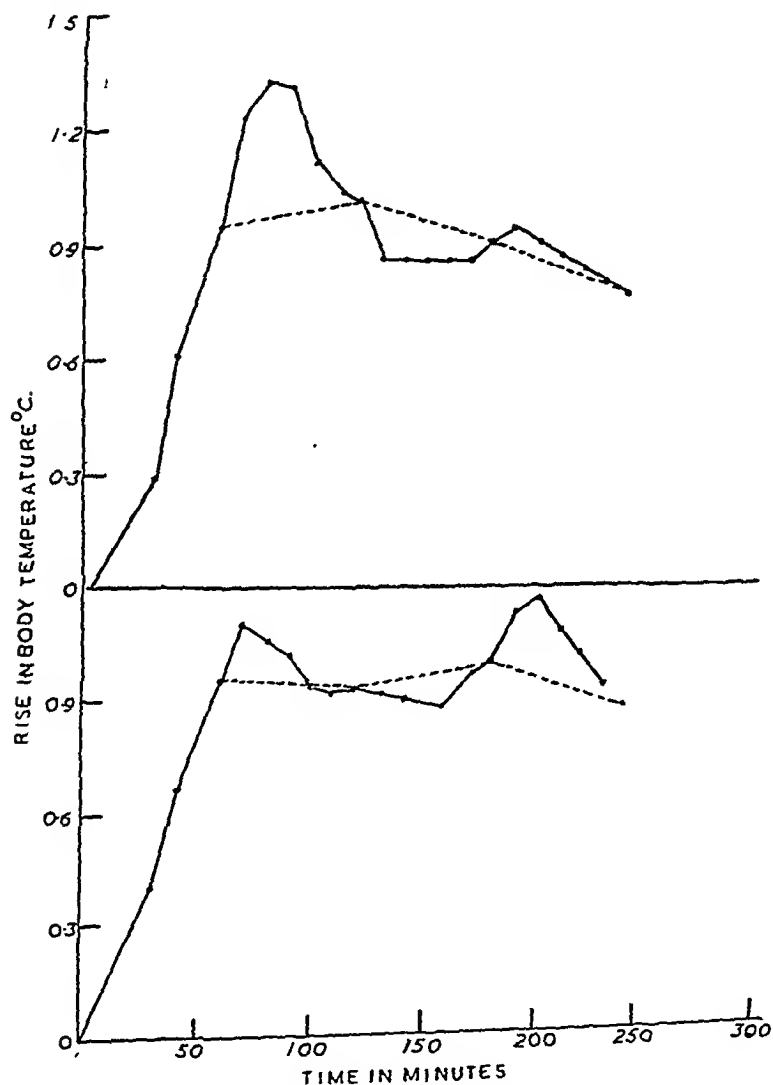


FIG. 2.—Showing effect of method of temperature determination on the shape of the fever curve.

———— Thermocouple method.
 - - - - - Thermometer method.

double-peak curve in a repeat of that test. Four explanations can be advanced for the differences in the shapes of the curves.

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1. That pyrogen in a whole culture containing the organism and its metabolic products (such a whole culture has been found to stimulate the double-peak reaction) is both dissolved in the medium and contained within the bacterial cell. Here the first rapid rise in temperature may be produced by the pyrogen dissolved in the medium, and the second fever by the slow liberation of pyrogen from the bacterial cells as they are broken down in the blood-stream. The cell-free filtrate therefore should cause the immediate reaction only, and a suspension of the cells the delayed reaction.

2. That each of the three types of fevers is stimulated by a different substance.

3. That the immediate response fever is caused by one type of pyrogen, the delayed response by another type, and the double-peak response by a mixture of the two substances.

4. That the actual dosage of pyrogen is the controlling factor and that by varying the dose, the shape of the curve can be altered.

These explanations have been investigated in the experiments described below.

EXPERIMENTAL

A culture of *Proteus vulgaris* was grown for 4 weeks at 37°C. in gelatin hydrolysate synthetic medium and the following experiments carried out:

1. Samples of the whole culture were sterilised by autoclaving at 115°C. for 30 minutes and tested on two different groups of 5 rabbits at a dose level of 0.002 ml./kg. of body-weight. Both tests showed the double-peak type of fever, one of which is shown in Figure 3.

2. Samples of the viable culture were filtered free of bacterial cells by passage through Berkefeld filter candles and the filtrate sterilised by autoclaving at 115°C. for 30 minutes. This filtrate was clear to the eye and no cells could be detected on microscopical examination. At a dose of 0.002 ml./kg. of body-weight, similar to the dose of the whole culture, this filtrate stimulated only the single peak fever of the immediate response type, as is shown in Figure 3.

3. Samples of the autoclaved whole culture were centrifuged at 4,000 r.p.m. for 45 minutes, and the supernatant liquid decanted and retained. The cells were then washed 3 times with pyrogen-free saline, separated by centrifuging each time, and the washings discarded. The washed cells were suspended in pyrogen-free saline and the cell-count adjusted to approximately the same as that of the original culture, as determined by Brown's Opacity Tubes. Both the supernatant and the suspension of washed cells were tested. The suspension of cells stimulated the single-peak fever of the delayed type and the supernatant liquid the double-peak fever (Figure 3).

From the results of this experiment it is seen that the same culture can produce all three types of fever depending on the state of the sample when injected, that the fraction stimulating the immediate response, the cell-free filtrate of the viable culture, can be separated from that causing the delayed response (the washed cells) and that the combination of the

two fractions, i.e., the whole culture, causes the double-peak type of fever. This then tends to discount the theory that there are three separate substances and, also, the theory that the dosage of pyrogen is the important controlling factor; further proof of this latter point is that removal of the cells has little effect on the first peak but removes the second peak.

The fact that the supernatant liquid from the centrifuged sample caused

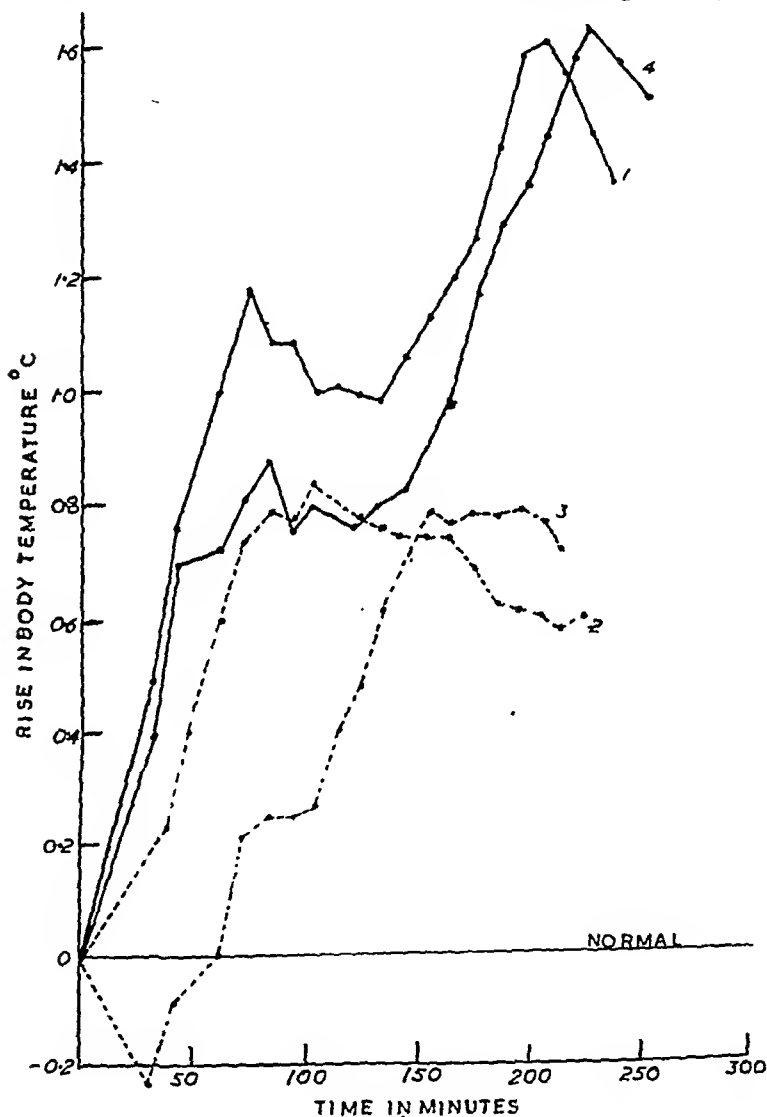


FIG. 3.—Showing different fever curves stimulated by modifications of the same culture of *Proteus vulgaris*.
 Curve 1.—Whole culture. Curve 2.—Cell-free filtrate.
 Curve 3.—Washed bacterial cells.
 Curve 4.—Centrifuged supernatant of sterilised culture.

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the double-peak type of reaction was unexpected, if this supernatant liquid were really cell-free, as it appeared to be when examined, the hypothesis that the double-peak fever is attributable to the same pyretic substance being present in the medium and in the bacterial cells is not tenable. Since the other results of the experiment supported the hypothesis it was possible that the centrifuged supernatant liquid still contained sufficient cells to cause the second peak. The above experiments were therefore repeated with additional refinements, mainly to find how many cells, if any, were necessary to stimulate the second peak, or

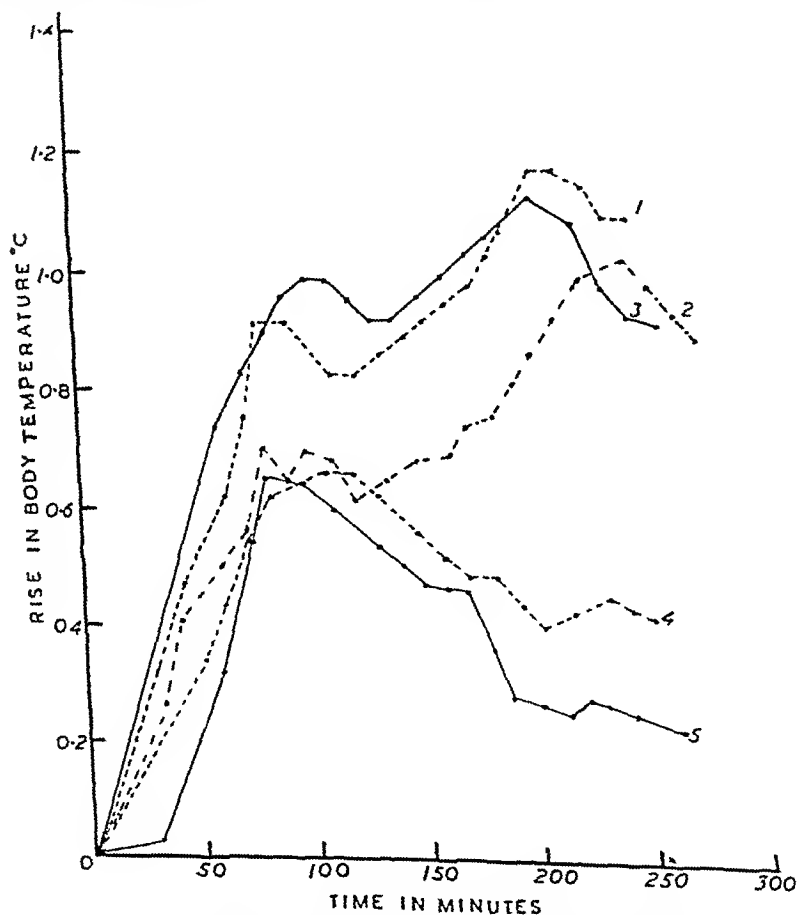


FIG. 4.—Elimination of second peak by reducing the dose.

Curve 1.—Dose in ml./kg. of body-weight.
 Curve 2.—0.02
 Curve 3.—0.004
 Curve 4.—0.0002
 Curve 5.—0.0002

whether it was possible to stimulate the double-peak type of fever by injection of a completely cell-free solution.

Escherichia coli was grown on a gelatin hydrolysate medium at 37°C. for 5 weeks and samples of this culture tested as follows:—

1. Samples of the whole culture were sterilised by autoclaving at

115°C. for 30 minutes; these were tested on groups of five rabbits in doses of 0.02, 0.004, 0.002 and 0.0002 ml./kg of body weight. The curves for these experiments are shown in Figure 4. The larger doses of 0.02 and 0.004 ml./kg. of body-weight stimulated the double-peak fever but those of 0.002 and 0.0002 ml./kg. of body-weight only the single-peak.

2. Samples of the viable culture were filtered free of cells by filtration through a Berkefeld filter candle, and the filtrate sterilised by autoclaving at 115°C. for 30 minutes. A dose of 0.02 ml./kg. of body-weight was tested on groups of 5 rabbits on 3 separate occasions, only the single-peak fevers of the immediate response type were stimulated. The curve for

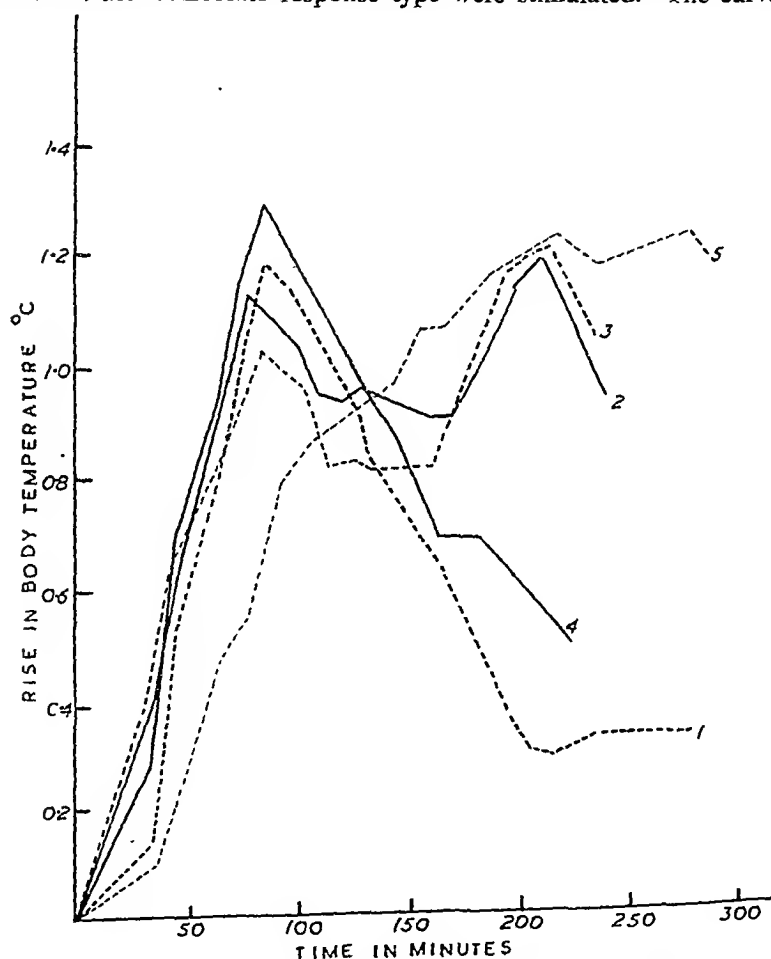


FIG. 5.—Showing different fevers stimulated by modifications of the same culture of *Escherichia coli*.

- Curve 1.—Filtrate of viable culture.
- Curve 2.—Filtrate of autoclaved culture.
- Curve 3.—Centrifuged supernatant of autoclaved culture.
- Curve 4.—Centrifuged supernatant of viable culture.
- Curve 5.—Washed bacterial cells.

one test is shown in Figure 5 (curve 1). However, in the previous set of experiments on *P. vulgaris*, the supposedly cell-free supernatant which unexpectedly gave rise to the double-peak reaction was prepared by sterilising the culture before the cells were removed by centrifugation and, to bring the filtration experiments into line with this, samples of the whole culture of *E. coli* were first sterilised by autoclaving and then filtered free of bacterial cells. A dose of 0.02 ml./kg. of body-weight of this filtrate was tested on groups of 5 rabbits on 2 separate occasions and the double-peak type of fever was obtained (Figure 5, curve 2). A dose of 0.002 ml./kg. of body-weight of the whole culture would contain more cells than a dose of 0.02 ml./kg. of body-weight of the filtered culture, but such a dose of the whole culture does not stimulate the double-peak fever and the filtrate does; it would appear, therefore, that, the few cells possibly present in the filtrate would not cause the double-peak. Therefore of the 4 theories advanced, the most probable one is that the solutions stimulating the double-peak of reaction contain two pyretic substances, one causing the immediate rise in body temperature and the other a delayed rise, a mixture of the two causing the double rise. By the fact that the second peak is either eliminated or reduced by removal of the cells, it would seem that most of the substance stimulating the second peak is within the cell, whereas that stimulating the first peak is mainly dissolved in the medium.

3. The tests on the centrifuged supernatant liquid were also carried out using the supernatant liquid of both the viable and the sterilised whole culture. In all cases the centrifuging was at 3,750 r.p.m. for $3\frac{1}{2}$ hours in an attempt to produce complete deposition of the cells, and no cells could be detected on microscopical examination. In four tests, each at a dose of 0.02 ml./kg. of body-weight, the supernatant liquid of the previously sterilised whole culture caused the double-peak fever, one of which is shown in Figure 5 (curve 3). The supernatant of the viable culture at the same dose stimulated only the single-peak fever (Fig. 5, curve 4). These results therefore support those obtained for the filtrate. The cells obtained by centrifugation of the sterilised whole culture were washed 3 times with pyrogen-free water and finally diluted with the required amount of saline solution to bring the cell count back to approximately that of the original culture, and tested at a dose of 0.02 ml./kg. of body-weight. The fever developed was of the delayed type as was found in the previous experiment (Figure 5, curve 5).

A third series of experiments were carried out, again on *P. vulgaris*, using a seven-day culture:

1. Three doses of the whole culture, 0.02, 0.002 and 0.0002 ml./kg. of body-weight, were tested. The two higher doses stimulated the double-peak reaction and the dose of 0.0002 ml./kg. of body-weight the single-peak reaction, showing again that the second peak can be diluted out more readily than the first.

2. Filtrates from both the viable and the sterilised cultures were tested, that of the autoclaved culture stimulated a definite double-peak response and that of the viable culture the single-peak reaction.

Summarising these results we have, that the second peak of a double-peak fever is more readily diluted out than the first, that the cell-free filtrate or centrifuged supernatant stimulates the single-peak type of fever if the cells are removed before autoclaving, and the double-peak type if the whole culture is autoclaved before removal of the cells, and that the washed cells of the centrifuged culture cause the production of the delayed fever. From the results therefore it appears that there are two pyretic substances, one causing the immediate fever and the other the delayed fever, and a mixture of both the double-peak fever. However, it also appears that the substance causing the second peak might be formed by the autoclaving of the cells. If the culture is autoclaved while the cells are present this substance is found in the medium, whereas, if the cells are first removed before autoclaving, the substance is usually absent. To clarify this point, a further set of experiments were carried out on a seven-day culture of *Proteus vulgaris*. The whole culture was divided into three parts, the first was sterilised by autoclaving at 115°C. for 30 minutes, the second by heating at 60°C. for 1 hour and the third by addition of 0.3 per cent. of chlorocresol. At a dose of 0.02 ml./kg. of body-weight all three solutions caused the double-peak type of reaction. The actual quantity of chlorocresol injected into the rabbits was 0.1 mg. and would have little effect. Thus the substance stimulating the double-peak fever is not formed by autoclaving the cells. Another possible explanation is that in the viable culture the substance causing the first peak is mainly dissolved in the medium and that stimulating the second peak mainly within the cell, and if there is any of this latter substance in the medium it is not usually present in sufficient quantity to stimulate the second peak. Autoclaving however increases the quantity of this substance in the medium sufficiently that the cell-free solution can stimulate the double-peak type of fever. This explanation is further supported by the fact that *Serratia marcescens* (*Bacillus prodigiosus*) stimulated the double-peak fever whether the culture was sterilised before or after removal of the cells, showing that the second-peak substance is not an artifact produced by autoclaving the cells. It may be assumed that in this case the second substance passes more readily from the cell into the medium than it does in either *Escherichia coli* or *P. vulgaris*.

The organisms examined which have stimulated the double-peak fevers are *P. vulgaris*, *P. inorganii*, *Ps. fluorescens*, *Ps. aeruginosa*, *S. marcescens*, *S. keilensis*, *E. coli*, *Eberthella typhosa*, *B. mycoides* and *Staphylococcus aureus*. On the other hand it cannot be claimed that other organisms tested in the course of this work which did not stimulate the double-peak type of reaction do not produce the necessary substance, as it may not have been present in the solutions tested in sufficient quantity to stimulate the second peak.

PRODUCTION OF DEPRESSANT SUBSTANCE BY SOME ORGANISMS AND THE EFFECT THIS HAS ON THE FEVER CURVE

Reactions, following injection of cultures of *P. vulgaris* and *Ps. fluorescens*, were obtained from time to time which differed from the

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usual type of fever reaction. In these cases the body temperature commenced to rise sharply in the first hour, as it would in the usual fever reaction, but this was followed by a rapid fall in body temperature to well below normal. In one of the tests the average fall for five rabbits was 1.34°C . below the initial temperature (Figure 6), one of the rabbits actually showing a fall of 3.4°C . within $1\frac{1}{2}$ hours of the injection. In the more severely affected rabbits of the groups, the other symptoms were signs of general collapse, the hind quarters were paralysed and there was no control over urination, the animals could not stand and the head

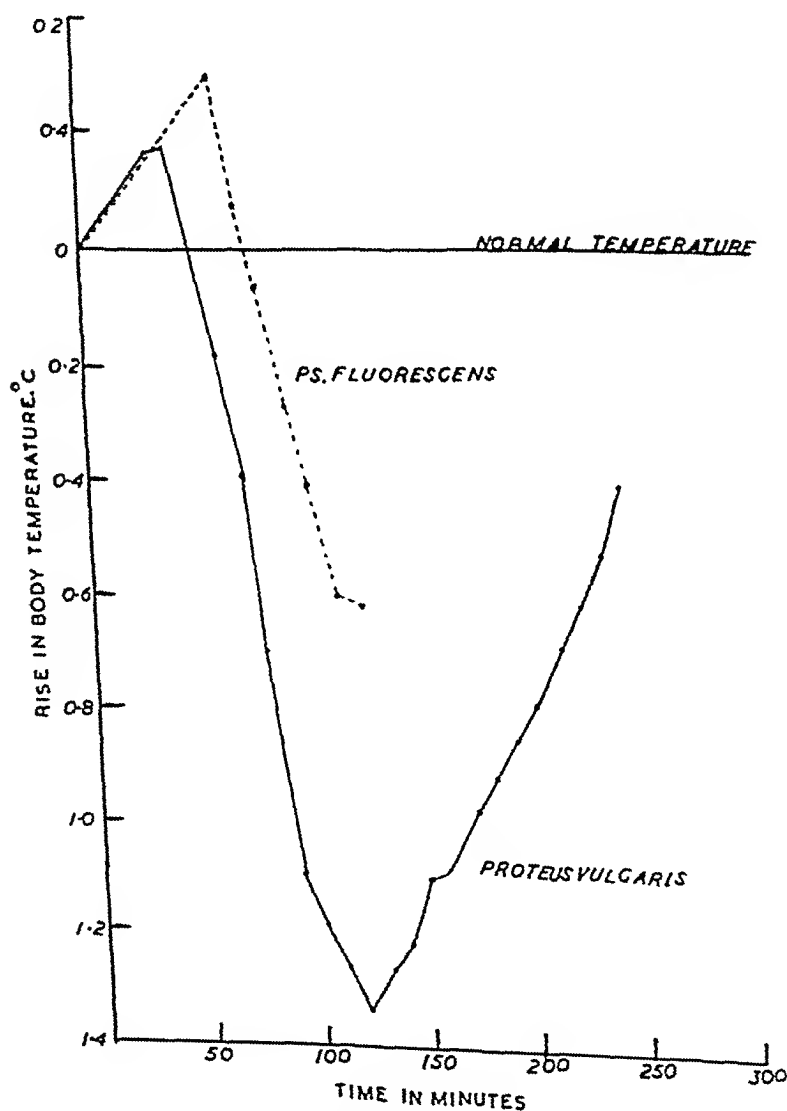


FIG. 6.—Fall in body temperature caused by a depressant substance present in cultures of *Ps. fluorescens* and *Proteus vulgaris*.

could only be moved slightly. These symptoms appeared in $1\frac{1}{4}$ to $1\frac{3}{4}$ hours after the injection. Two hours after the injection the animals usually began to recover from this state, the temperature rose slowly and the animal was soon able to stand and move about. The symptom which was apparent in all of the animals injected, whether severely affected or not, was the loss of appetite, the animal refusing food for a number of days after the experiment.

The depressant effect was not repeated when further samples of the same cultures were retested, and it was also observed that when the reaction occurred, the first rabbit in each group to be injected was usually the most severely affected. As the solution to be injected is heated to approximately body temperature before injection, it was realised that if the depressant were very volatile it might be slowly driven off and, therefore, the solution injected into the first rabbit might contain a higher concentration of the depressant substance than that injected into the following rabbits. On this assumption experiments were carried out on a seven-day culture of *P. vulgaris*. Samples of the whole culture, in sealed ampoules, were sterilised by autoclaving at 115°C . for 30 minutes, and tested in doses of 0.02 and 0.002 ml./kg. of body-weight. In these tests the solution was diluted, placed in an open beaker and heated to 30° to 40°C . for about 10 minutes before injection. This solution produced the usual fever reaction (Figure 7), no depressant action or loss of appetite being observed. Similar samples of this culture were tested in doses of 0.04, 0.02 and 0.01 ml./kg. of body-weight, this time taking care during the preparation of the solution to prevent loss of any volatile substance, and the solution was not heated before injection. Here the depressant action was apparent at a dose of 0.04 ml./kg. of body-weight, there being a distinct fall in body temperature and development of paralysis (Figure 7). The rabbits receiving the other two doses, although not showing the fall in body temperature nor the paralysis, lost appetite for food for a few days. Thus the substance causing this fall in body temperature and loss of appetite is volatile, and since it is present in the cultures autoclaved in sealed ampoules, it appears to be relatively stable towards heat.

Modifying effect of depressant on the fever.—Figure 8 shows how the height of the fever stimulated by a culture may be modified by this depressant substance. When the solution was heated before injection it caused the usual type of fever, the modifying effect of the depressant is seen in the fevers stimulated by the solution injected without previous heating, the temperature rose sharply in the first hour but instead of continuing to the usual peak in $1\frac{1}{2}$ hours, it either fell sharply, or tended to remain at the point reached. Such a reaction, in which the depressant effect is not very obvious, might lead to the wrong conclusions concerning the pyrogenic activity of the solution. The depressant was inferred to be present in these solutions, for, although the temperature of the animals did not fall below normal, they refused food for a number of days following the experiment. It is clear that when testing cultures of bacteria it is advisable to heat the solutions before injection and the result of any

experiment which is followed by the refusal of the animals to eat should be examined for signs of the modifying effect of the depressant, and the experiment repeated if necessary.

This depressant might also account for the distinct fall in body temperature following the injection of the washed cells of *P. vulgaris*, as is seen in Figure 3. In this delayed fever the temperature fell before beginning to rise, and this reaction seems to be similar to the reactions referred to by Seibert⁷ and Hort and Penfold⁶.

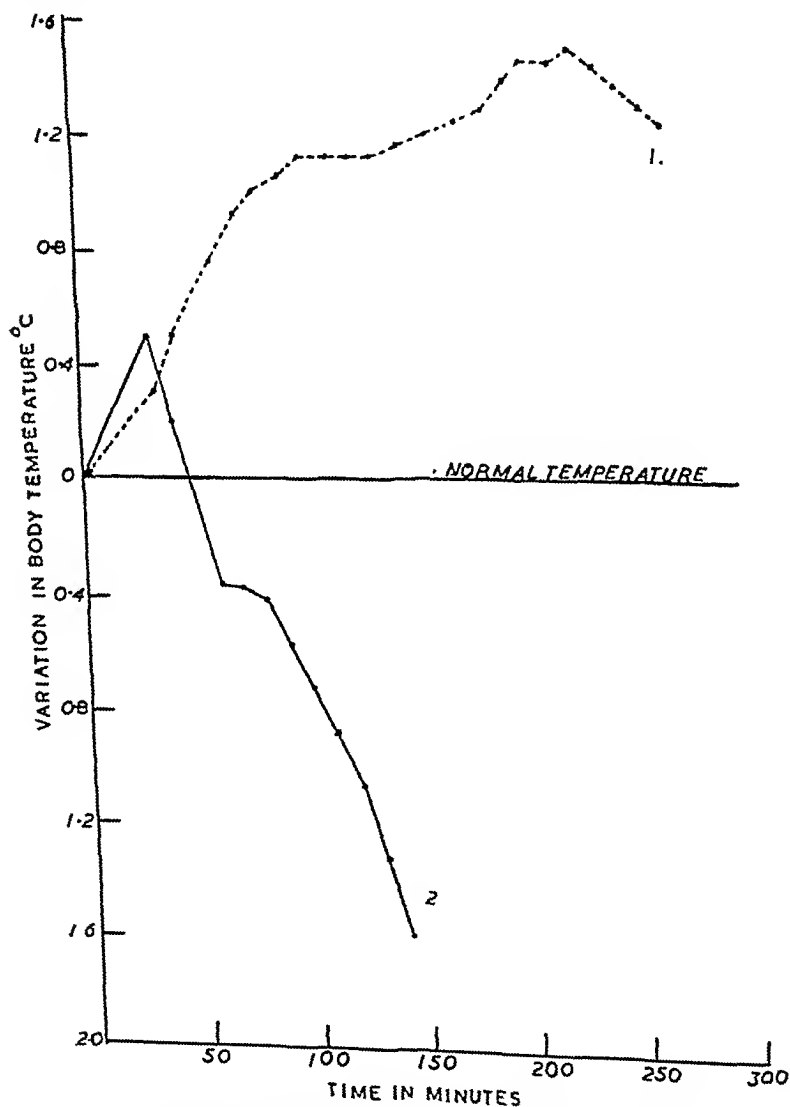


FIG. 7.—Showing effect of heating solution before injection.
Curve 1.—Solution heated before injection.
Curve 2.—Solution not heated before injection.

Variations in stability to heat.—The stability of pyrogen to heat has been investigated by Seibert³ and Banks⁷, both concluded that it was a thermostable substance. In a previous paper, however, we showed that a cell-free filtrate of a 48-hour culture of *P. vulgaris* showed an initial rapid loss in activity when heated at 120°C., but that the solution was still pyrogenic after 4 hours at 120°C.

A further 48-hour and a 31-day culture of *P. vulgaris* and also cultures of *Ps. aeruginosa*, *B. subtilis* and *M. tetragenes* of 3, 2 and 1 weeks incubation at 37°C. respectively, have since been examined for stability

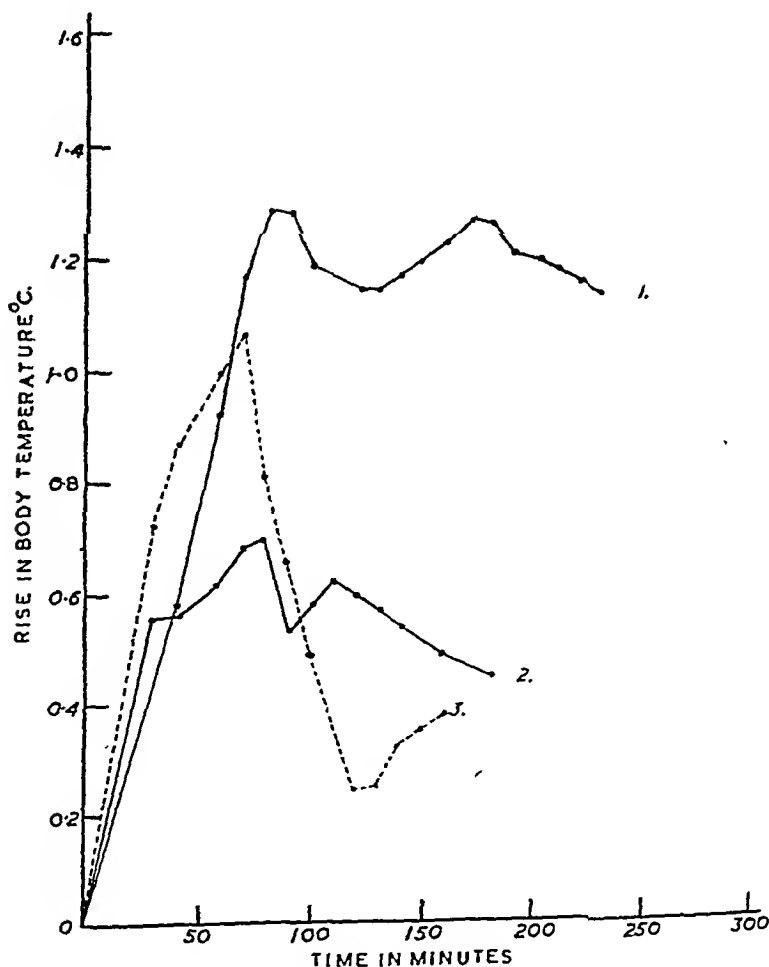


FIG. 8.—Modifying effect of depressant on the fever stimulated by a whole culture of *Proteus vulgaris*.

Curve 1.—Dose—0.002 ml./kg. of body weight, no depressant effect.
 Curve 2.—Dose—0.02 ml./kg. of body weight, depressant effect.
 Curve 3.—Dose—0.005 ml./kg. of body weight, depressant effect.

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to heat at 120°C. As the method of heating at 120°C. by autoclaving, as described in the previous paper, is not easily controlled or reproduced, the cultures were filtered free of bacterial cells, sealed in thick-glass ampoules and immersed in an oil-bath maintained at $120 \pm 1^\circ\text{C}$. The reaction of each solution was previously adjusted to pH7, it was not considered necessary to add buffering agents to the solution, as it was the buffered medium in which the organisms had been grown. Samples of each of the cultures were heated at 120°C. for 30 minutes, 1 hour, 1½ hours and 2 hours. The strengths of the unheated cultures were calculated by extrapolation of the log. concentration against time curve, which is a straight line. Table I shows the results for this set of experiments.

It would seem, from the results, that the rate of loss inactivity of cultures of *P. vulgaris*, *B. subtilis* and *Ps. aeruginosa* is somewhat similar, in that approximately 50 per cent. is destroyed after 30 minutes and 95 per cent. after 2 hours. The activity of the *M. tetragenes* culture, on the other hand, was much more difficult to reduce, only 20 per cent. of the original activity being destroyed after 2 hours at 120°C.

TABLE I
SHOWING THE RATE OF DESTRUCTION OF THE PYROGEN FROM
PS. AERUGINOSA, *B. SUBTILIS* AND *M. TETRAGENES*

Heat Treatment		<i>Pseudomonas aeruginosa</i>		<i>Bacillus subtilis</i>		<i>Micrococcus tetragenes</i>	
Temp °C	Duration hours	Units/ml	Percentage destroyed	Units/ml	Percentage destroyed	Units/ml	Percentage destroyed
Extrapolation		214	0	19	0	9.1	0
120	½	75	65	9	52.6	8.75	3.8
120	1	51	76	4.4	76.8	8.0	12.6
120	1½	26.9	87	2.4	87.4	7.5	17.6
120	2	10.6	95	0.9	95	7.3	19.8

<i>Proteus vulgaris</i>								
		48-hour culture		48-hour culture		31-day culture		Average percentage destroyed
60	1	46	0	57.5	0	825	0	0
120	½	31	33	42.5	38	362	56	42
120	1	6.75	85	21.9	76	400	52	71
120	2	2.0	96	6.2	93	41	95	94
120	4	0.75	98	1.9	98	14	98	98

Linear relationship between the log. of concentration and time.—By determining the percentage of the original concentration left at each stage, and plotting the logarithm of this against time, a linear relationship was found to exist for all four cultures, which satisfied all points within the experimental error of the quantitative test, i.e. about 15 per cent. Wylie and Todd⁸. In the case of *Proteus vulgaris*, a batch was heated at 120°C. for 4 hours, but this point deviated in all three experiments from the linear relationship of the other points.

Figure 9 shows that the same straight line, the "calculated line of best fit" in this case, can be drawn to represent the rate of destruction of the pyrogen produced by *P. vulgaris*, *Ps. aeruginosa* and *B. subtilis* within the

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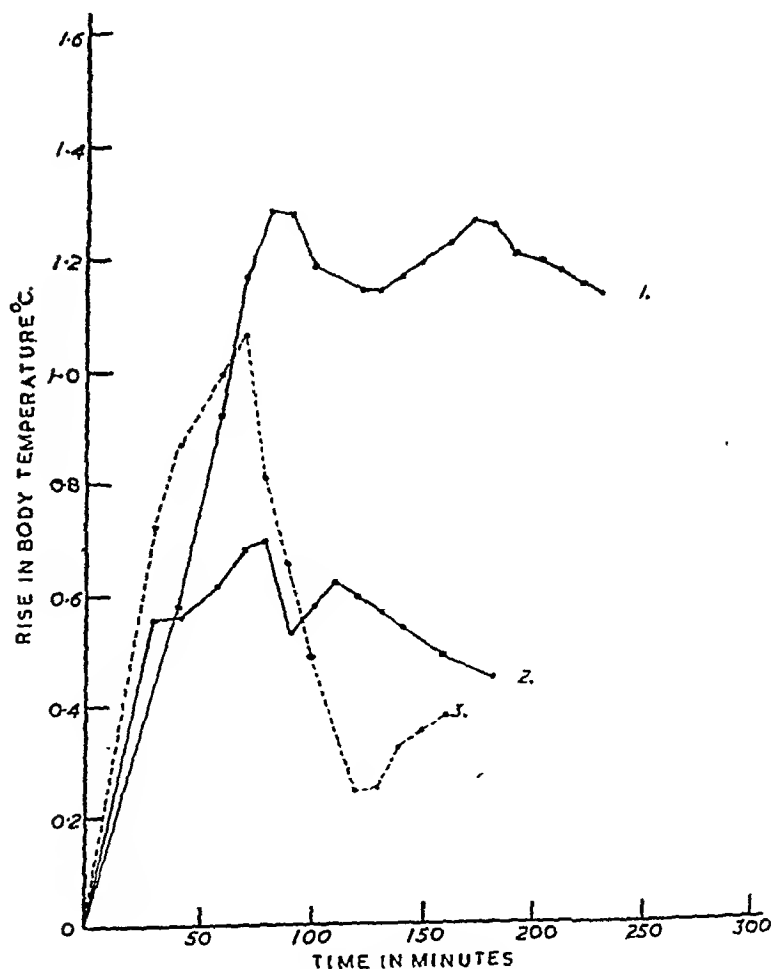


FIG. 8.—Modifying effect of depressant on the fever stimulated by a whole culture of *Proteus vulgaris*.

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 Curve 2.—Dose—0.02 ml./kg. of body weight, depressant effect.
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PYROGEN FROM VARIOUS SOURCES

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120	4	0.75	98	1.9	98	14	98	98

Linear relationship between the log. of concentration and time.—By determining the percentage of the original concentration left at each stage, and plotting the logarithm of this against time, a linear relationship was found to exist for all four cultures, which satisfied all points within the experimental error of the quantitative test, i.e. about 15 per cent. Wylie and Todd⁸. In the case of *Proteus vulgaris*, a batch was heated at 120°C. for 4 hours, but this point deviated in all three experiments from the linear relationship of the other points.

Figure 9 shows that the same straight line, the "calculated line of best fit" in this case, can be drawn to represent the rate of destruction of the pyrogen produced by *P. vulgaris*, *Ps. aeruginosa* and *B. subtilis* within the

experimental error of the quantitative test. The results for *M. tetragenesis* although still linear are quite different.

Summarising these results, we find that the pyrogen present in the cell-free filtrate of cultures of *Ps. aeruginosa*, *P. vulgaris* and *B. subtilis* show very similar rates of destruction. *M. tetragenesis*, on the other hand, seems to produce a pyretic substance of totally different stability. How far these results would agree with those obtained by the use of pure pyrogen is unknown, and continuation of the work on these lines, for purposes of comparing the stability of pyrogen produced by different organisms, would be of little value unless carried out on the purified substances. The results obtained here fulfil the desired requirements, in that they give an indication of how much pyrogen may be lost during any heating process, and also of the degree of severity of treatment permissible in the isolation of pyrogen.

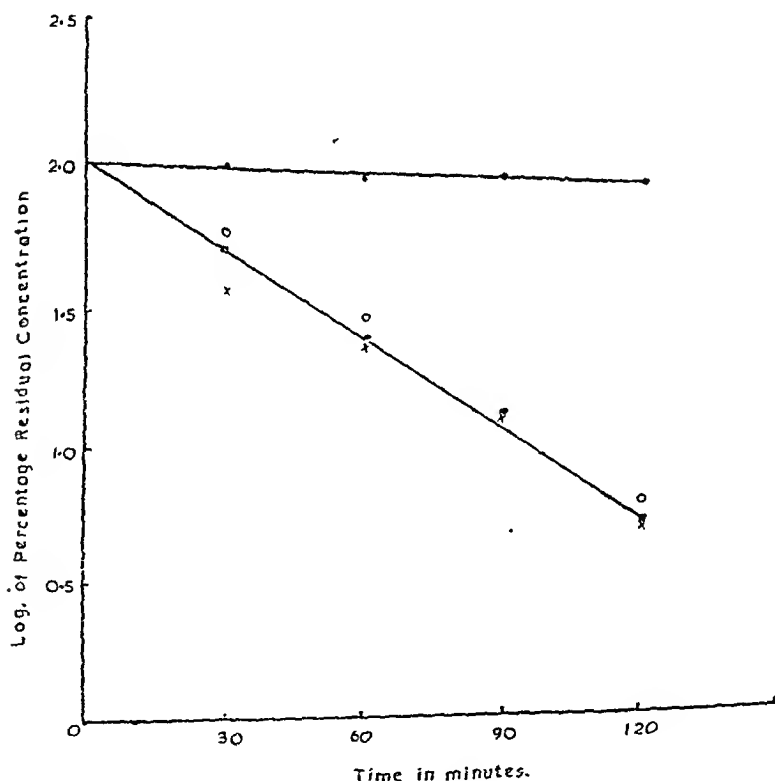


FIG. 9.—Showing linear relationship between logarithm of residual concentration and duration of heating at 120°C.

Upper graph *Micrococcus tetragenesis*.
 Lower graph ○ *Proteus vulgaris*.
 ● *Bacillus subtilis*.
 × *Pseudomonas aeruginosa*.

SUMMARY

1. Three distinct types of fever curves can be stimulated by injection of modifications of the same bacterial culture.

2. Injection of a sterilised whole culture causes a double-peak fever; the cell-free filtrate or supernatant liquid of a culture stimulates a single-peak fever, rising to a peak in an average time of 86 minutes if the cells are removed before autoclaving, and a double-peak reaction if the culture is autoclaved before removal of the cells; the fever caused by injection of a suspension of washed bacterial cells is a single-peak fever of the delayed reaction type, reaching its peak in 3 to 4 hours.

3. The results show that certain bacteria probably produce two pyretic substances, one of which, in the viable culture, is dissolved in the medium and stimulates the single-peak fever of the immediate reaction type, and the other is contained mainly in the bacterial cell and stimulates the single-peak fever of the delayed reaction type, a mixture of both causing the double-peak fever.

4. *Proteus vulgaris* and *Ps. fluorescens* can produce a volatile depressant substance, which either lowers the body temperature to below normal, or prevents the pyrogen from causing a rise in body temperature. This depressant causes general paralysis if injected in sufficiently large doses, in smaller quantities the most obvious effect is the loss of appetite. It has been shown that any pyrogen test followed by the rabbits refusing to eat for a few days should be examined for other effects of the depressant substance, and should be repeated, taking care to heat the solution to between 30° and 40°C. for about 10 minutes before injection.

5. An investigation of the stability of pyrogen to heat at 120°C. showed that *Ps. aeruginosa*, *B. subtilis* and *P. vulgaris* produce pyretic substances of similar stability, whereas *M. tetragenes* produces a much more thermostable substance.

6. The results from the investigation of the stability showed that the pyrogens of *P. vulgaris*, *Ps. aeruginosa* and *B. subtilis* are sufficiently labile to require care when using heat during isolation, as approximately 95 per cent. is destroyed after 2 hours at 120°C. Finally it must be emphasised that further investigation into these points would be of little value unless carried out on the pure pyretic substances isolated from various bacterial sources.

This work was carried out during the tenure by one of us (D.W.W.) of a Carnegie Scholarship and a Wellcome Research Fellowship to the trustees of which we wish to express our thanks.

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DISCUSSION

The paper was read by Mr. Wylie.

The CHAIRMAN said that the subject of pyrogens appeared to be more complicated than had been thought originally; he assumed the authors were intending to continue their work, and he would suggest to them that they should try chromatography as a method of separating pyrogens.

PROFESSOR H. BRINDLE (Manchester) said that the presence of the depressant seemed important, and it should be taken note of in testing for pyrogens. Had the authors distilled their cultures in order to make certain that this depressant substance was volatile?

In Figure 4 the authors used dilutions in order to show that the second factor could be diluted out. This introduced difficulties as regards quantitative work. If the Figure was correctly printed it would appear that there was a considerable difference between the action of two exactly similar doses of 0.02 ml., and it also seemed that 0.004 ml., only of 1/5 of this dose would produce a greater response than the whole dose.

DR. G. E. FOSTER (Dartford) asked whether the authors had been able to confirm the destruction of pyrogens in solution on keeping as had been reported at the Torquay Conference. His Company had been asked by an outside organisation to do some pyrogen tests for them, and one of the substances had made the rabbits' temperature fall very markedly. They had subsequently found out that the material submitted to them was a salicylate, which was an antipyretic. Had the authors any experience of testing for pyrogens in antipyretics?

MR. E. H. REID (Dagenham) asked whether the culture of *B. prodigiosus* had been treated in the same way as that of *P. vulgaris*, i.e., by autoclaving at 115°C. for 30 minutes, by heating at 60°C. for an hour or by the addition of 0.3 per cent. of chlorocresol. If not, the possibility that the double peak was due to the sterilisation procedure could not be excluded.

MR. G. E. SHAW (Runcorn) said that in work on scour in calves and on infantile gastro-enteritis they had found that by extracting a culture of organisms by boiling they could obtain a toxic substance which did not come out into the culture medium.

This substance, which was only present in extremely small amounts, had been found to contain about 15 per cent. of carbohydrate. When concentrated it produced a temperature reaction in calves with a characteristic gastro-intestinal lesion. It also killed rabbits, guinea-pigs, and mice. It would also act as a rather feeble antigen, but one could prepare an antiserum against it. The organism was grown on a medium containing ammonium sulphate, protein acid hydrolysate and glucose, and in deep culture the medium was aerated. After about 20 hours the count was about 3,000 million organisms per ml. Could Mr. Wylie give the total count of the culture he had used, and also the viable count? Was the pyrogen reaction simply a form of antigen-antibody reaction, or was it the effect of a pyretic chemical? He thought that different pyrogens might be of a similar type, all producing an antigen

PYROGEN FROM VARIOUS SOURCES

tissue reaction. If they shook up pyrogen with an adsorbent such as zeolite, did they remove the constituent responsible for the rapid effect, or that causing the more slow reaction?

MR. J. A. MYERS (Bradford) asked if the authors had compared their results with the temperature curves of patients after transfusions. Quite frequently a rise of temperature was ascribed to the presence of pyrogens, but it might be due to the patient's clinical condition. Such comparisons might roughly indicate whether a patient's rise of temperature was due to something within the solution, such as bacteria, or due to some clinical interference related to the patient's condition.

MR. G. R. MILNE (Glasgow) said that the presence of a depressant substance was of special interest in connection with blood transfusion. In a well organised transfusion unit large quantities of transfusion fluids were produced with no trouble from pyrogenic reactions, but with blood and blood products, no matter how careful one was, there were always spells when reactions occurred. Unfortunately when they occurred careful records were not usually made, and it was therefore difficult to find an explanation for them. Many samples of blood had been returned to his depot to be tested for bacterial contamination and all the reports were negative. He was interested in the report of the depressant substance, because the condition of the patient might be such that the ratio of the amount of depressant substance to that of the pyrogen would turn the balance.

MR. D. W. WYLIE, in reply, said that they were not directly concerned with the depressant substance and had merely investigated it to find how it was produced, and how it could be eliminated before they tested the pyrogenic culture. The substance could be distilled off. The double peak response curve upset the quantitative test to some extent, but they had found the test still accurate provided one regarded only the first peak. The experiments in Figure 4 were not necessarily carried out at doses which were in the quantitative range; therefore one might get one-fifth of a dose stimulating response similar to that of the full dose.

In this paper and in the previous one, they had given results of the stability of pyrogen at 120°C. They had tested various solutions for pyrogen but not, so far, antipyretic solutions. Their culture of *B. prodigiosus* had been sterilised by autoclaving at 115°C. for 30 minutes. In this case the intracellular substance passed into the medium.

They had done the total counts and viable counts asked for by Mr. Shaw and he would send them on. They did not seem to be very important. They did not think the pyrogenic response was an antibody antigen reaction. They had not compared their fever counts with patient's curves, but the rise of temperature in rabbits had been similar.

PROFESSOR J. P. TODD, the joint author of the paper, said he would like to add that the depressant substances were being investigated, and Mr. Wylie's successor had started on the work.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

***m*- and *p*-Cresol in their Mixtures, Colorimetric Analysis of.** S. A. Savitt, A. M. Goldberg, and D. Othmer. (*Anal., Chem.*, 1949, **21**, 516.) The method used was based on the production of coloured nitroso-phenols. The cresols were dissolved in a potassium hydroxide-glacial acetic acid buffer solution, and sulphuric acid followed by sodium nitrite solution was added. After standing, alcoholic ammonium hydroxide was added, the solution allowed to stand overnight, and the colour read photoelectrically using a violet light filter (492 m μ). Standard curves of the colour transmissions of the nitroso-solutions are given together with the calibration curves for known concentrations of *m*- and *p*-cresol and their mixtures. The rate of absorption of water by the cresols in the atmosphere was determined and a graph is given for *m*- and *p*-cresols; it was concluded that the small amount of water absorbed over the initial 15 minutes of exposure would have no effect on the precision and accuracy of the analytical procedure. Numerous unknown samples were analysed and gave results which were reproducible to within 1 per cent. If phenol or *o*-cresol were present, it was found to be necessary to remove it by an efficient distillation and rectification prior to analysis for the binary mixture itself.

R. E. S.

Iron, Volumetric Determination of. W. D. Cooke, F. Hazel and W. M. McNabb. (*Anal. chem.*, 1949, **21**, 643.) Solutions of ferric salts, acidified with either sulphuric or hydrochloric acid, were reduced by treating with liquid zinc amalgam. After separation of the amalgam, the residual ferric ion was reduced by the addition of a few drops of chromous chloride solution, the chromous ion reactions being followed by a low potential redox indicator, phenosafranine (oxidation-reduction potential, -0.28 volt). Complete reduction of the ferric ion was indicated by the colour change of the indicator from pink (oxidised form) to colourless, while the reverse colour change indicated complete oxidation of the excess chromous ion by atmospheric oxygen. No evidence of oxidation of ferrous iron by air was observed under the conditions of the experiment, thus eliminating the necessity of maintaining an inert atmosphere during liquid zinc amalgam reductions. In the actual titration of a ferric salt liquid amalgam was added followed by sulphuric or hydrochloric acids. After 1 minute carbon tetrachloride was added, the amalgam was removed (the reactions being carried out in a separating funnel), 2 drops of phenosafranine indicator were added followed by chromous chloride (usually 4 to 5 drops) until the pink colour of the indicator disappears and a clear green tint was visible; at this stage the solution was swirled until the pink colour reappeared. Phosphoric acid and diphenylamine sulphonate were then added and the titration was completed with standard potassium dichromate solution. An indicator correction was found to be necessary.

R. E. S.

Lævulose, Determination of. D. T. Englis and J. E. Miles. (*Anal. chem.* 1949, **21**, 583.) The fact that lævulose was found to produce consider-

able colour, while dextrose produced practically no colour. when treated with the Folin-Denis phosphotungstic-phosphomolybdate reagent, was used as a basis for the colorimetric determination of levulose in the presence of dextrose. A 20 per cent. solution of trisodium phosphate was added finally in place of the usual sodium carbonate solution, and a series of trial determinations indicated that 10 minutes was the optimum time for heating in a water-bath at 100°C. Graphs are given for the extinction values (determined photoelectrically) of the blue colours produced by solutions of levulose and dextrose of known concentrations when treated under the conditions recommended. In order to determine the amount of levulose in a mixture a procedure is outlined which necessitates the determination of total reducing sugars by another method. Using the known values, the percentage of levulose can then be determined since the blue colour produced by the dextrose in the Folin-Denis reaction is relatively low in intensity.

R. E. S.

Potassium, Colorimetric Determination of, by Folin-Ciocalteu Phenol Reagent. M. A. M. A b u l - F a d l. (*Biochem. J.*, 1949, 44, 282.) The method depends on the fact that alkaline solutions of cobalt salts, in the presence of a trace of an amino-acid (glycine or alanine), reduce the phosphomolybdic-phosphotungstic acid phenol reagent to a blue colour, the intensity of which is directly proportional to the amount of cobalt present, and hence, if potassium has been precipitated as cobaltinitrite, to the amount of potassium in the original solution. Details are given for the precipitation of the potassium from the serum as cobaltinitrite and for the washing of the precipitate. The precipitate is dissolved in water, and glycine, sodium carbonate and Folin's solutions added when, after standing at 37°C. for 10-15 minutes, the blue colour is read photoelectrically. The reduction of the phosphotungstic-phosphomolybdic acids by cobalt salts could not be effected in the absence of amino acids; the intensity of the blue colour was dependent on the amino-acid concentration, the optimum colour being obtained with 0.2 to 0.5 M-glycine. Amino-acids of higher molecular weight than alanine gave a blue colour with the phenol reagent in the absence of cobalt, the intensity of the colour depending on the molecular weight. The method can conveniently be carried out on 0.2 ml. serum instead of 0.5 ml. required for previous methods.

R. E. S.

Riboflavin in Foodstuffs and Biological Material, Fluorimetric Estimation of. E. K o d i c e k and Y. L. W a n g. (*Biochem. J.*, 1949, 44, 340.) The material under examination is first extracted on a water-bath with 0.1N hydrochloric acid, the proteins being precipitated by the use of metaphosphoric acid and/or digestion with takadiastase (for starchy materials.) The extract is washed with chloroform in acid solution and oxidised with saturated potassium permanganate solution followed by treatment with hydrogen peroxide to decolorise the remaining permanganate. At this stage the extract is adjusted to pH 6, methyl alcohol is added to clarify if necessary, and fluorimetric readings are taken using a blue filter for the incident light and a yellow filter between the solution and the photocell. A sensitive photocell-galvanometer circuit is used and in the blank riboflavin is reduced to a non-fluorescent compound with a neutralised solution of sodium dithionite. Several samples were analysed in 8 to 12 replicates and the coefficient of variation ranged from 2.2 to 4.6 per cent. The accuracy of the method decreases with decreasing concentration of riboflavin in the

material examined. Details of procedure are given together with a standard curve for pure riboflavine.

R. E. S.

ORGANIC CHEMISTRY

Aloin. Characters and Reactions of. C. L. Harders. (*Pharm. Weekbl.* 1949, 84, 250, 273.) The solubility of aloin from Curaçao aloes was determined for a number of solvents at 20°C., with the following results:—

Solvent	Solubility
Chloroform	1:33333
Carbon tetrachloride	1:20000
Ether	1: 3333
Ether (saturated with water) ..	1: 1818
Ethyl acetate	1: 56.4
Amyl alcohol	1: 523.6
Dichlorhydrin	1: 34.8
Dioxan	1: 13.3
Water	1: 155.4
Methyl alcohol	1: 13.2
Ethyl alcohol	1: 46

Impure (commercial) aloin has a higher solubility in water. The molecular weight of aloin, determined cryoscopically in ethyl urethane, was found to be 413. By hydrolysis in the absence of oxygen, no anthranols are formed, as under these conditions they give condensation products. When oxygen is present hydrolysis gives an emodin. A preliminary oxidation is also necessary in order to remove a pentose from the molecule. The fresh sap of the plant contains no emodin. Results suggesting the presence of an easily split glucoside of an anthraquinone derivative are due to the presence of mucilage, derived from the leaf, and to the action of oxygen on aloin. A new compound which was isolated from the sap is the cause of the intense colour. This compound crystallises in needles (m.pt. 179°C.), is readily soluble in benzene, and has a molecular weight of 230.5. For the determination of aloin in aloes four methods are suggested: chlorination (Leger); determination of pentose (Goldner); determination by persulphate (Seel) and determination with ferric chloride. Details of the last method are as follows: 10 mg. of aloes is dissolved in 0.5 ml. of water and treated with a solution of 0.3 g. of ferric chloride and 38 g. of calcium chloride (2H₂O) in 20 ml. of 0.5N hydrochloric acid. The mixture is boiled under a reflux for 45 minutes, diluted with an equal volume of water, and filtered through glass wool. The filtrate is shaken out successively with 25, 25, and 15 ml. of ether. The residue on the glass wool is dissolved in 4N sodium hydroxide and poured into a slight excess of N hydrochloric acid. The suspension obtained is also shaken out with ether. The combined ethereal solutions are washed successively with 5 ml. of water, 2.5 ml. of phosphate buffer (pH8) and 5 ml. of water. The solution is then shaken with 20 per cent. ammonia until no more emodin is extracted, the volume of the ammoniacal solution being finally made up to 250 ml. The emodin is determined colorimetrically, using as standard a solution prepared in a similar manner from pure aloin. Good samples of Curaçao aloes contain nearly 40 per cent. of aloin.

G. M

Myristicin, Synthesis of. V. M. Trikojus and D. E. White. (*J. chem. Soc.*, 1949, 436.) The constitution of myristicin as 1-methoxy-2:3-methylenedioxy-5-allylbenzene has been confirmed by synthesis (preliminary report *Nature*, 1939, 144, 1016). Allylation of pyrogallol 1-methyl ether gave

two monoallyl ethers, the lower boiling ether being characterised as pyrogallol 1-methyl 2-allyl ether. Pyrolysis of this ether gave 2:3 dihydroxy-1-methoxy-5-allylbenzene which on treatment with methylene iodide and potassium carbonate gave myristicin, identical with the natural product obtained from oil of nutmeg. This identity was established by comparison of samples of tetrabromomyristicin, isomyristicin, and tetrabromoisomyristicin prepared from natural and synthetic myristicin. Pyrolysis of the higher boiling monoallyl ether (pyrogallol 1-methyl-3-allyl ether) and methylenation of the product produced myristicin in smaller yield.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Vitamin A, Refining of Oils containing. By J. H. Burgoyne and F. A. Burden. (*Nature*, 1949, 163, 722.) It has been thought that alkali-refining of fish-liver oils containing vitamin A decreases their potency because of the absorption of vitamin A by soaps formed *in situ*. Experiments with a number of oils seem to indicate that just the opposite is the case. The neutralised oil, far from losing anything of its original potency, extracts to a large extent vitamin A from the soap formed during the neutralisation. In oils with high potency and high acid value this increase can be quite substantial, as illustrated by the following example.

	Weight (g.)	Vitamin A Potency (I.U./g.)	Total content of Vitamin A. (I.U.)
Original oil	24.50	120,000	2,940,000
Neutralised oil	18.75	140,000	2,625,000
Oil in soapstock	5.75	44,000	253,000
(by difference)			

The loss of vitamin A during the alkali-refining is due to some neutral oil being carried away with the soap-stock, while the potency of the neutralised oil has increased. As the latest refining methods allow of a loss closely approaching the theoretical Wesson loss, it seems beyond doubt that alkali-refining provides the most economical and simple method of purifying oils containing vitamin A

S. L. W.

BIOCHEMICAL ANALYSIS

p-Aminosalicylic Acid in Body Fluids, Determination of. J. P. Newhouse and W. Klyne. (*Biochem. J.*, 1949, 44, VII.) The method employs diazotisation and coupling at room temperature instead of at 0°C. Oxalated blood, plasma or cerebro-spinal fluid (0.2 ml.) is measured into a test-tube containing 6.7 ml. of water, the contents of the tube are shaken, allowed to stand for 3 minutes and trichloroacetic acid (0.6 ml. of a 25 per cent. w/v solution) is added; after shaking, the mixture is allowed to stand for 15 minutes and is then filtered through a small Whatman paper (No. 40 or 42) into a graduated centrifuge tube. To the clear filtrate (5.0 ml.) concentrated hydrochloric acid (1.5 ml.) and a 1 per cent. solution of sodium nitrate (0.2 ml.) are added, the tube is shaken for 30 to 40 seconds and 1.0 ml. of ammonium sulphamate reagent (consisting of 2.0 g. ammonium sulphamate in 50 ml. of glacial acetic acid and 50 ml. of water) is added. The resulting solution is shaken for 10 seconds and 1.0 ml. of naphthylethylenediamine

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ORGANIC CHEMISTRY

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dihydrochloride solution (0.2 per cent.) is added. *p*-Aminosalicylic acid gives a purple colour attaining its maximum intensity after standing for 15 minutes at room temperature and stable for at least 3 hours. The colour intensity is measured with a photoelectric photometer, using a yellow-green filter (e.g. Ilford No. 605) and is compared with that obtained from a standard representing 10 mg. of *p*-aminosalicylic acid per 100 ml. of blood. In the preparation of the standard, 5.0 ml. of a stock solution (containing 183.9 mg. of sodium *p*-aminosalicylate dihydrate/l.) is diluted to 100 ml., 2.0 ml. of this solution, 2.6 ml. of water, and 0.4 ml. of trichloroacetic acid solution are mixed and treated as the 5.0 ml. of blood filtrate. In setting the instrument to zero a blank consisting of 0.4 ml. of trichloroacetic acid solution and 4.6 ml. of water is used and is treated as the 5.0 ml. of blood filtrate; a blank determination on blood is not usually necessary. The colour developed is directly proportional to the *p*-aminosalicylic acid concentration in the range 0 to 10 mg./100 ml. of blood, but this linear relationship does not hold strictly at higher concentrations. When known quantities of *p*-aminosalicylic acid (1 to 20 mg./100 ml.) were added to normal blood or to cerebro-spinal fluid the following recoveries were obtained: blood 77 to 94 per cent., mean 85 per cent.; cerebro-spinal fluid 90 to 100 per cent., mean 94 per cent. Duplicates agreed within about 0.3 mg./100 ml. Streptomycin did not interfere in the determination

R. E. S

Heparin, A New Method for the Assay of. H. Kjems and H. Wagner. (*Acta Pharmacol. Toxicol.*, 1948, 4, 155.) A method for the assay of heparin is described based on the fact that, under suitable conditions, the clotting times in mixtures of varying amounts of heparin and constant amounts of oxalated ox plasma and thrombin are gradually prolonged between 20 and 60 seconds, so that a smooth curve is obtained for clotting time plotted against amount of heparin. 0.07 ml. of heparin standard solution and 0.13 ml. of 0.9 per cent. sodium chloride solution are pipetted into a test tube, 1 ml. of oxalated ox plasma is added and the contents are mixed by swinging the test tube before the addition of 0.1 ml. of thrombin solution. The test tube is at once placed in a water thermostat at 37°C., and during the first 5 sec. the test tube is vigorously moved in order to produce rapid temperature equilibrium; it is then swung to and fro with a quiet pendulum movement, being raised slightly over the horizontal position each time it is moved to the left. The moment of clotting is noted when the mixture does not run but slides down the wall of the test tube. Sodium chloride solution (0.9 per cent.) must be added to the thrombin solution until a clotting time of 30 to 35 sec. is attained. The oxalated plasma is made by centrifuging fresh ox blood with 10 per cent. of a 5 per cent. potassium oxalate solution, stored at 0°C. for at least 24 hours, and again centrifuged immediately before use. A standard curve of clotting times using known amounts of heparin is prepared and the strength of an unknown solution of heparin can thus be determined. A number of variables in this assay have been studied including the preparation of the plasma, the volume of the heparin solution and the influence of pH. The presence of a hitherto unknown factor taking part in the coagulation of the blood is shown.

R. E. S.

Œstrogens, A New Method for the Separation of Androgens from, and for the Partition of Œstriol from, the Œstrone-Œstradiol Fraction. N. B. Friedgood, J. B. Garst and A. J. Haagen-Smit. (*J. biol. Chem.*, 1948, 174, 523.) The application of ultra-violet absorption spectrophoto-

metry to the quantitative determination of the urinary oestrogens was investigated with a view to developing an objective physical method for their accurate determination. The causes of inaccuracies of the methods in current use were investigated, including the following aspects of the problem: the spectrophotometric identification and quantitative micro-determination of crystalline oestrogens; the detection by spectrophotometric assay of gross errors in current methods for extraction and partition of oestrogens; studies on the ultraviolet absorption of substances used for the extraction and partition procedures; the separation of the phenolic oestrogens from the androgen steroid fraction; the separation of urinary oestrogens from the other urinary phenolic substances by steam distillation; and the micro-Girard separation of oestrone from oestradiol. A new method was developed for the extraction, partition and quantitative determination of crystalline oestrone, oestradiol and oestriol. The oestrogens were separated from the androgens by partition between N potassium hydroxide and a mixture of 1 volume of solvent ether and 18 volumes of carbon tetrachloride. Benzene and disodium hydrogen phosphate were used for the separation of the oestrogens into strongly and weakly phenolic fractions because 0.3M sodium carbonate carries one-third of the oestradiol over into the oestriol fraction. A specially designed apparatus made it possible to reduce to a minimum the number of transfers of extracts and residues. The sensitive ultra-violet spectrophotometric method which was used for the assay of crystalline oestrone, oestradiol and oestriol gave consistent results and crystalline oestrogens were recovered quantitatively.

L. H. P.

Phenazone in Biological Materials, Estimation of. B. B. Brodie, J. Axelrod, R. Soberman, and B. B. Levy. (*J. biol. Chem.*, 1949, 179, 25.) Two methods for the determination of this compound in biological materials are described. One procedure involves the extraction of phenazone from an alkaline solution of the biological material (organ tissues and faeces are prepared for analysis by emulsification in acid) with chloroform, and the evaporation of the solvent. The residue is dissolved on dilute sulphuric acid, sodium nitrite is added and the resulting 4-nitrosophenazone is determined by spectrophotometric measurement of the light absorption at 250 m μ . The other procedure, suitable for plasma, involves the estimation of the phenazone directly in the plasma filtrate after removal of the protein with zinc hydroxide. A table is given, showing satisfactory recovery experiments in both procedures when known amounts of phenazone were added to tissues and to plasma. Reproducible analyses of plasma and urine stored in the refrigerator for several days showed that the compound is stable in biological fluids. Distribution experiments between plasma and ethylene dichloride indicated that the substance estimated was actually phenazone. Many drugs found in plasma did not interfere. Sulphadiazine interferes since it absorbs light at 350 m μ , the absorption being increased by the addition of nitrous acid.

R. E. S.

Urobilinogen, Ehrlich's Aldehyde Test for. T. M. Wilson and L. S. P. Davidson. (*Brit. med. J.*, 1949, 1, 884.) Owing to the considerable variation in the reagents used, and to the fact that there are numerous substances other than urobilinogen which can give colour reactions with *p*-dimethylaminobenzaldehyde the aldehyde test lacks specificity. With the various reagents in current use the final concentration of hydrochloric acid varies between 0.35 and 2.5 per cent. (excluding Watson's reagent in

which the hydrochloric acid is immediately neutralised by sodium acetate) and the final concentration of *p*-dimethylaminobenzaldehyde varies between 0.06 and 0.6 per cent. Ehrlich's rosindole reagent, which is still used by some workers, has the composition: *p*-dimethylaminobenzaldehyde 4 g., absolute alcohol 380 ml., and concentrated hydrochloric acid 80 ml. Apart from urobilinogen the substances most liable to give rise to a reddish-pink colour when urine is tested with Ehrlich's aldehyde reagent are indole, the precursors of two indole pigments—indirubin and urorosein—and porphobilinogen. The reaction is more sensitive if urines are tested while fresh and warm, and precautions detailed should be rigidly observed. Further work is required to determine the optimal quantities of *p*-dimethylaminobenzaldehyde and hydrochloric acid to be used and the relative amounts of reagent and urine to be employed in the test.

S. L. W.

CHEMOTHERAPY

***p*-Hydroxybenzenesulphonamides.** K. A. Jensen and S. A. K. Christensen. (*Acta chem. scand.*, 1949, 87, 207.) The following compounds were prepared by diazotising the corresponding *p*-amino compounds and boiling the diazonium chlorides so obtained with water:—2-(*p*-hydroxybenzenesulphonamido)-thiazole, m.pt. 221 to 222°C., 2-(*p*-hydroxybenzenesulphonamido)-benzthiazole, m.pt. 292°C., 2-(*p*-hydroxybenzenesulphonamido)-5-methylthiazole, m.pt. 231°C., 2-(*p*-hydroxybenzenesulphonamido)-5-methyl-1:3:4-thiadiazole, m.pt. 217 to 218°C., 6-methoxy-8-(*p*-hydroxybenzenesulphonamido)-quinoline hydrochloride, m.pt. 268°C., N-(3:4-dimethylbenzoyl)-*p*-hydroxybenzenesulphonamide, m.pt. 187°C., 6-(*p*-hydroxybenzenesulphonamido)-coumarin, m.pt. 230 to 231°C., *p*-hydroxybenzenesulphonylguanidine, m.pt. 160 to 162°C., *p*-hydroxyphenyl-2-aminothiazolyl(5)-sulphone, m.pt. 260°C., *p*-hydroxyphenyl-2-aminothiazolyl(5)-sulphone hydrochloride m.pt. 247°C. These compounds in a concentration of 1 in 5000 had no bacteriostatic action on *Diplococcus pneumoniae* (type 1), *Eberthella typhosa*, *Staphylococcus aureus* and *Escherichia coli*.

G. R. K.

Salicylic Acid, Some Substituted Amides of. K. A. Jensen and S. C. Linholt. (*Acta chem. scand.*, 1949, 87, 205.) Six amides were prepared by treating the appropriate amino compound in pyridine solution with acetylsalicyloyl chloride and removing the acetyl group by hydrolysis. The amino compounds used were 2-aminothiazole, 2-aminopyridine, 2-amino-5-methyl-1:3:4-thiadiazole, sulphanilamide, sulphathiazole and *pp'*-diaminodiphenylsulphone. When tested against *Diplococcus pneumoniae* (type 1), *Eberthella typhosa*, *Staphylococcus aureus* and *Escherichia coli*, they were without bacterial effect in a concentration of 1 in 5,000.

G. R. K.

PHARMACOLOGY AND THERAPEUTICS

iso-Amidone, a New Analgesic Drug Analogous to. P. Ofner, R. H. Thorp and E. Walton. (*Nature*, 1949, 163, 479). 6-Piperidino-4:4-diphenyl-5-methyl-3-hexanone (I) (the piperidyl analogue of isomidone) has been synthesised by a route involving the preparation of a nitrile $\text{Ph}_2\text{C}(\text{CN})\cdot\text{CHMe}\cdot\text{CH}_2\text{NC}_5\text{H}_9$ (II) as an intermediate. The constitution of the nitrile (II) was confirmed by exhaustive methylation and identification of the product, while work is proceeding with a view to isolating other isomers

of (I) which may have been formed in the synthesis. The piperidyl analogue of isoamidone prepared showed the smallest degree of undesirable side-actions of any of the active analgesic drugs yet studied. It showed the same effects in acute animal toxicity experiments as amidone or isoamidone, death in all cases resulting from acute cardiac failure; all these compounds differed from morphine since they were more toxic upon rapid intravenous injection. In man "piperidyl isoamidone" (I) in doses of 12.5 mg. produced no appreciable side-actions. After a 25mg. dose some of the volunteers described a sensation of warmth, were flushed and slightly dizzy, although the symptoms were less pronounced than those observed with other analgesic drugs. There was no significant effect upon the cardio-vascular system. The drug produced analgesia similar in duration to that of amidone, and showed sufficient promise to warrant extensive examination and clinical trials, particularly in obstetrics. Recent work has produced a nitrile, isomeric with (II), which yielded 6-piperidino-4:4-diphenyl-3-heptanone; preliminary results on this compound using young rats indicated that this ketone was approximately twice as active as morphine analgesically, yet only equal to it as a respiratory depressant.

R. E. S.

Curare and Erythroidine Alkaloids, a Biological Method for Determination of. E. P. Pick and G. V. Richards. (*Proc. Soc. exp. Biol., N.Y.*, 1949, 67, 329.) Groups of 5 to 10 white mice, weighing 18 to 20 g. each, are injected subcutaneously with 0.5 mg. of morphine sulphate. Restlessness and the typical tail reflex occur generally within 5 to 10 minutes and persist for 2 hours or more. Curare and erythrina preparations, dissolved in 0.85 per cent. sodium chloride solution, are injected intraperitoneally in a volume not exceeding 0.5 ml. into animals exhibiting the typical morphine reaction. A positive effect appears within 5 to 10 minutes and is characterised by disappearance of the excitement phenomena and relaxation of the tails. This period lasts usually for 10 to 25 minutes and is followed by a gradual re-appearance of restlessness and tail phenomena. The median effective dose and standard error for crystalline *d*-tubocurarine chloride is $2.8 \pm 0.2 \mu\text{g.}$ for *Strychnos* curare $24.0 \pm 2.0 \mu\text{g.}$ for dihydro- β -erythroidine bromide $44.0 \pm 3.0 \mu\text{g.}$ and for intocostin 20.0 ± 2.0 millunits. Much higher doses of other drugs, including quaternary alkaloids, are necessary to antagonise the effect of small amounts of morphine in mice. Myanesin, in doses of 8 mg. per mouse, despite its curare-like action on the striated muscles, is not able to depress the tail reflex in morphinised mice.

S. L. W.

Curarising and Anti-curarising Action, Prolongation of. H. J. Chase, B. K. Bhattacharya and J. L. Schmidt. (*J. Pharmacol.*, 1949, 95, 95) Measurable curare action has been demonstrated in rabbits by the "head-drop" assay method to be present 48 hours after the subcutaneous injection of a suspension containing 30 mg. (200 units)/ml. of *d*-tubocurarine chloride in a peanut oil and 4.8 per cent. beeswax vehicle. Suspension of neostigmine in the vehicle failed significantly to prolong the action of neostigmine. The duration of the anti-curare effect of neostigmine intravenously and subcutaneously, when measured in rabbits, corresponds closely with the duration of clinical effects of this drug seen in the treatment of myasthenia gravis. The duration of the anti-curare action of a single intravenous injection of di-isopropylfluorophosphonate solution containing 1 mg./ml. was also determined in rabbits. The maximum antagonism to curare occurred following a dose of 2 mg./kg. and the duration of this action for 12

was shown that the pellets implanted subcutaneously are absorbed as slowly as fat-soluble hormones. The dose used in the implant varied from 20.0 to 92.7 I.U./kg. of bodyweight, except in one case in which 450 units were given. The calculation of a daily absorption of 1 per cent. was confirmed in these cases, which assures a maximum duration of activity of the implant of about 100 days. Massive absorption has never taken place and there is therefore no danger of hypoglycæmic shock. Protamine zinc insulin alone is absorbed as slowly as the cholesterol complex, so that even if the pellets were to break there would be no risk. This method of administering insulin not only abolishes injections but may achieve better results since the continuous action of the implant imitates the effect of an artificial endocrine pancreas.

S. L. W.

Iron Therapy of Anæmia, Intravenous. J. A. Nissim and J. M. Robson. (*Lancet*, 1949, 256, 326.) Saccharated iron oxide was prepared from the following analytical grade reagents: ferric chloride hexahydrate, anhydrous sodium carbonate, sodium hydroxide and sucrose. It was found that there was a linear relationship between toxicity and the effect of heating, as measured by the physical characteristics of the substance produced, thus making it possible to select the best samples for intravenous injection. The best sample was found to have LD50 300 mg. or 15 ml./kg. of a 2 per cent. solution, as compared with the 180 mg. or 9 ml./kg. of the original sample reported on. This new preparation has been repeatedly given intravenously to several patients in doses of 300 mg. of elemental iron without toxic reaction, and it is possible that even larger doses might be tolerated. Compounds of iron with glucose or fructose instead of sucrose were found to form much more easily and require less heat for their preparation, but they were also more toxic, having an LD50 of about 150 mg. or 7.5 ml./kg. in mice. Samples prepared according to the method described by Stack and Wilkinson invariably precipitated on autoclaving, probably due to the formation of ferric glucosate. True saccharated iron oxide is much more resistant to heat and shows hardly any breakdown when autoclaved.

S. L. W.

Iron Therapy, Intravenous. G. Hemmeler. (*Acta med. scand.*, 1949, 132, 364.) Iron given by intravenous injection has a much greater erythropoietic action than when given by mouth. Moreover iron may be administered in this manner with advantage to patients who are intolerant of iron by mouth or in whom there is faulty absorption of iron. Ferrous or ferric salts are not well tolerated by injection owing to the fact that the iron is present in the ionised form. If, on the other hand, the medicament is given in the form of a complex salt in which the iron is only slowly ionised in the system after injection secondary effects are less liable to occur. A non-ionised preparation which meets this requirement, namely, the sodium salt of ferri-di-(α -dioxo- β -dimethyl) butyrate, has been successfully used by the author in the treatment of iron-deficiency anæmia. The therapeutic dose of this preparation is well below the dose which is likely to give rise to toxic effects, and a dose of 40 mg. daily is well tolerated and cures even severe cases of iron-deficiency anæmia within 3 or 4 weeks. Intravenous iron therapy should not be used except in patients suffering from iron deficiency, since animal experiments show that iron in excess of that required for the synthesis of hæmoglobin is not eliminated but is stored in the liver and spleen.

S. L. W.

N-Methyloxyacanthine Iodide, Curariform Activity of. D. F. Marsh, D. A. Herring and C. K. Sleeth (*J. Pharmacol.*, 1949, 95, 100.) Oxyacanthine, isolated from *Berberis vulgaris*, has many structural components found in *d*-tubocurarine. A comparison of the pharmacological activity of its quaternary derivative, N-methyloxyacanthine iodide, in rats, rabbits, dogs and man, with that of *d*-tubocurarine chloride shows that it is one-half to three fourths as active as paralyzing agent. It differs however in that it possesses a weak atropine-like action. In trained dogs, paralyzing doses of *d*-tubocurarine chloride produce copious salivation, but paralyzing doses of N-methyloxyacanthine iodide produce no salivation. A similar effect occurs in man; during experiments with N-methyloxyacanthine, subjects experienced a dryness of the mouth which persisted for several hours after the experiment had terminated.

S. L. W.

Myanesin in the Treatment of Tetanus. M. H. A. Davison, A. B. Ward and E. A. Pask. (*Brit. med. J.*, 1949, 1, 616.) Hæmoglobinuria following the use of myanesin in tetanus has been reported in about 20 cases. Two cases are described. The first patient was treated mainly by sedatives and *d*-tubocurarine chloride and received 58 ml. of myanesin intravenously over a period of 60 hours. He recovered and had no hæmoglobinuria. The second, a much more severe case, was treated solely with myanesin, which efficiently controlled the spasms until hæmoglobinuria developed, after 265 ml. had been given, in 26½ hours. The myanesin was then discontinued; sedatives were given but were ineffective and the patient died 2 hours later. During these two hours hæmoglobinuria almost disappeared, and the kidneys were normal to the naked eye and on section. This supports the view that hæmoglobinuria may be temporary and might perhaps be disregarded. These cases indicate not only that myanesin acts efficiently in controlling the spasms of tetanus, without paralyzing the muscles of respiration, but that its use in massive doses will bear further investigation. Tolerance did not develop, which was in marked contrast to the reaction of the first case to sedative drugs.

S. L. W.

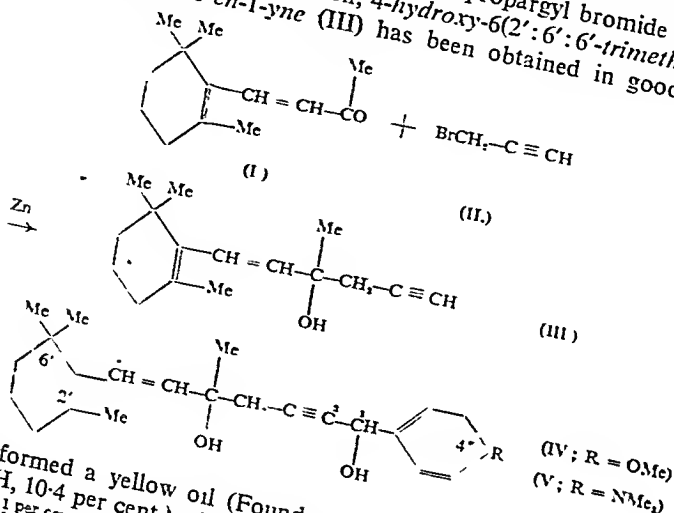
Thiourea in the Treatment of Thyrotoxicosis. G. T. Kent, R. A. Shipley and K. D. Rundell. (*Amer. J. med. Sci.*, 1949, 217, 627.) A series of cases of thyrotoxicosis were treated with daily doses of 0.1 to 0.3 g. of propylthiouracil or with thiourea in a similar dosage. The latter usually received also 15 minims daily of Lugol's solution of iodine. The clinical efficacy of the two substances was found to be of similar magnitude but thiourea was inferior to propylthiouracil in that it gave a higher incidence of side reactions, chiefly fever, which occurred in 16 per cent. of cases. The relapse rate in 27 patients, treated for from 3 months to 2 years before the drug was withheld, was 77 per cent.

H. T. B.

LETTER TO THE EDITOR

The Synthesis of

4-hydroxy-6(2':6':6'-trimethylcyclohex-1'-enyl)-4-methyl-hex-5-en-1-yne
 Sir,—By condensation of β -ionone (I) with propargyl bromide (II) employing a Reformatsky type of reaction, 4-hydroxy-6(2':6':6'-trimethylcyclohex-1'-enyl)-4-methyl-hex-5-en-1-yne (III) has been obtained in good yield. The



product formed a yellow oil (Found: C, 83.2; H, 10.4; C₂₄H₃₂O requires C, 82.7; H, 10.4 per cent.) which showed selective light absorption at $\lambda_{\max} = 232\text{m}\mu$, $E_{1\%}^{1\text{cm}} = 255$, and gave a violet-blue colour with the Carr-Price reagent. On catalytic microhydrogenation it absorbed 2.7 molar equivalents of hydrogen, the 1':2'-cyclohexenyl double bond evidently resisting hydrogenation because of its tertiary character. Zerewitinoff determinations revealed the presence of two active hydrogen atoms.

The structure assigned to (III) has been confirmed by reaction of its Grignard reagent with *p*-methoxybenzaldehyde when 1:5-dihydroxy-7(2':6':6'-trimethylcyclohex-1'-enyl)-1(4"-methoxyphenyl)-5-methyl-hept-6-en-2-yne (IV) Found: C, 77.8; H, 9.7; OMe, 7.6. C₂₄H₃₂O₃ requires C, 78.2; H, 8.8; OMe, 8.4 per cent. was obtained. Reaction with *p*-dimethylaminobenzaldehyde gave the corresponding 4"-dimethylamino-analogue (V) (Found: C, 78.4; H, 9.4; N, 3.7; C₂₅H₃₅O₂N requires C, 78.7; H, 9.2; N, 3.7 per cent.), characterised by selective light absorption at $\lambda_{\max} = 260\text{m}\mu$, $E_{1\%}^{1\text{cm}} = 470$.

Experiments on the conversion of (III) into vitamin A alcohol are in progress. The authors thank Dr. R. E. Stuckey and Mr. P. S. Stross for the absorption data, and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

Research Department,
 The British Drug Houses, Ltd.,
 London, N.1.
 10th October, 1949.

B. N. FEITELSON
 V. PETROW
 O. STEPHENSON.

NEW REMEDIES

The asterisk () after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.*

Diparcol* is a proprietary brand of diethazine hydrochloride or β -diethyl-aminoethyl-N-phenthiazine hydrochloride, a pinkish-white crystalline powder, almost odourless and having a bitter, numbing taste. M.pt. $175^{\circ}\text{C}.$; soluble 1 in 10 in water at $25^{\circ}\text{C}.$, in ethyl alcohol and in chloroform, but insoluble in ether. It is rapidly absorbed from the gastro-intestinal tract and is especially indicated in Parkinson's syndrome. It acts mainly upon hypertonia, treatment leading to disappearance of rigidity; tremor is reduced, and cramps and paræsthesia relieved. Treatment is started with from 2 to 5 0.05 g. tablets every 4 to 5 days until the most effective dosage is reached. Full improvement usually appears after 6 to 10 weeks of treatment. Untoward reactions include mental confusion, sometimes with nausea and vomiting, somnolence and occasionally exacerbation of symptoms. Diparcol is issued in sugar-coated tablets containing 0.05 and 0.25 g. in bottles of 100 and 500, and in 5 ml. ampoules.

S. L. W.

Disecron* is a compound solution containing 2.5 mg. of α -estradiol monobenzoate and 12.5 mg. of progesterone in 1 ml. of ethyl oleate, for intramuscular injection. It is indicated in the treatment of certain forms of secondary anæmia, the most satisfactory result being obtained in cases of less than 2 years' duration. It is most likely to be successful in secondary amenorrhœa of the emotional and environmental type. One injection is given intramuscularly on each of 2 consecutive days, succeeding 2-day courses being repeated at 28-day intervals. It is issued in boxes of 2, 10 and 25 ampoules each containing 1 ml., and in vials of 10 ml.

S. L. W.

Gluco-Thricil* is a combination of ephedrine 1 per cent. and tyrothricin 1 in 5000 in an isotonic dextrose solution containing cetyltrimethylammonium acetate as a solubilising and stabilising agent. It is indicated for the relief of nasal congestion in conditions such as the common cold, acute catarrhal rhinitis, allergic rhinitis and acute rhino-sinusitis. Tyrothricin is bactericidal for many Gram-positive organisms, including those commonly found in nasal secretions, and is bacteriostatic to certain Gram-negative organisms. Being isotonic with nasal secretions, it does not interfere with ciliary action and does not cause irritation or stinging. It is supplied in 1 fl.oz. bottles, with dropper.

S. L. W.

Neo-Octon* is a proprietary brand of isoamylaminomethylheptene hydrochloride, a synthetic antispasmodic with low toxicity and freedom from side-effects. It is indicated in conditions in which it is desired to relieve spasm of the smooth musculature, e.g., spastic conditions of the gastro-intestinal tract, ureter and bladder, dysmenorrhœa and biliary colic. Orally, 1 or 2 tablets (0.05 to 0.1 g.), or 10 to 20 drops of 5 per cent. solution, are given twice or three times daily; parenterally, 0.5 to 1 ml. (0.025 to 0.05 g.) is given twice or three times daily subcutaneously, intramuscularly, or if necessary, intravenously. Neo-Octon is issued in tubes of 10 or bottles of 100 and 500 tablets; in bottle of 10 ml. and 100 ml. of a 5 per cent. solution; and in boxes of 6 and 30 ampoules.

S. L. W.

REVIEW ARTICLE

THE CARDIOACTIVE GLYCOSIDES

BY ARTHUR STOLL

DR. RER. NAT. DR. MED. PHARM. ET SCI. TECH. H.C.

INTRODUCTION

THE impressive description given by William Withering in 1785 of the action of digitalis leaves upon the failing heart resulted in such a fundamental advance in the treatment of cardiac diseases that, even to-day, therapeutic measures are still based upon the experiences he recorded. In the intervening period, however, it has been possible to adapt the methods of treatment better to the various diseases of the heart and circulation than was originally the case.

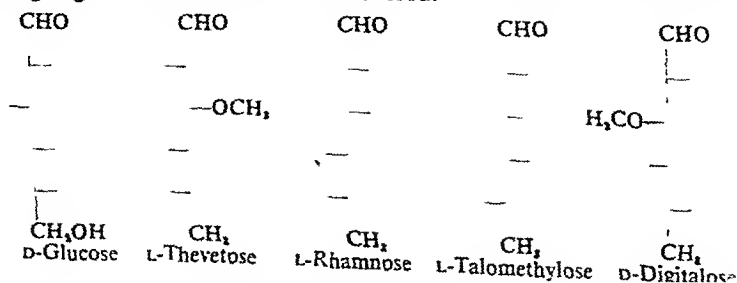
Since the time of Withering there has been introduced into therapy a whole series of cardioactive drugs fundamentally similar in effect to the leaves of *Digitalis purpurea*, but differing, for example, in their rapidity and duration of action.

The chemical investigation of the drugs from which the cardioactive glycosides are obtained also dates back to the middle of the 19th century. Particularly in the last two decades, however, it has made such rapid strides that to-day the constitution of practically every important cardiac glycoside is known, including the details of its steric configuration.

In this article, an attempt will be made to characterise the more important cardiac glycosides but without pursuing the long trail which led to their isolation and to the elucidation of their chemical constitutions. The main difference between modern processes of isolation and the earlier methods employed lies in the fact that account is now taken of the great sensitivity of the cardiac glycosides to high temperatures, to acids or alkalis, and to enzymatic cleavage, etc. Not only digitoxin but all the other cardiac glycosides known prior to 1930, with the exception of ouabain and scillaren A, are in fact, artificial products arising as the result of enzymatic cleavage from substances of a higher sugar content originally present in the plant. This reveals one reason for the difference in action repeatedly observed by physicians between the use of the older pure substances on the one hand and of the crude drugs on the other.

THE SUGARS OF THE CARDIOACTIVE GLYCOSIDES

One of the main characteristics of glycosides is the fact that they contain one or more sugar residues. In the cardiac glycosides, the following sugars have so far been discovered.



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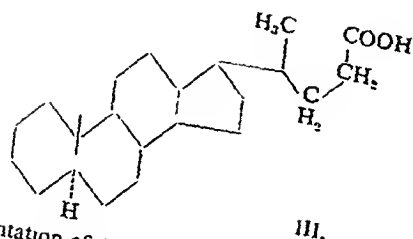
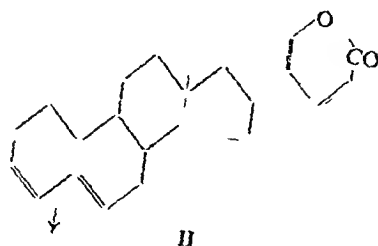
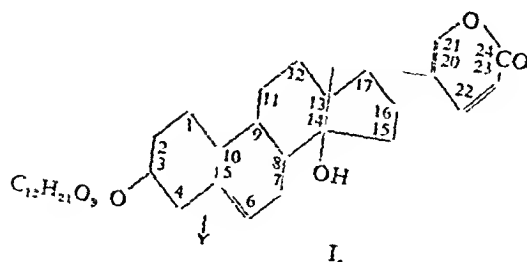
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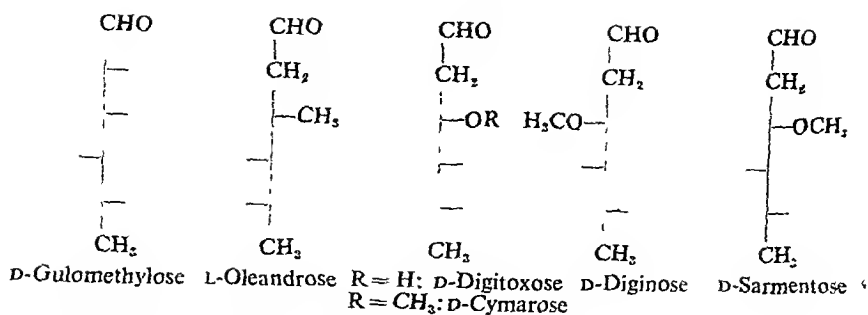
THE CARDIOACTIVE GLYCOSIDES

aglycone, scillaridin A, into a product of animal origin, allocholanolic acid⁵.



Schematic representation of the conversion of scillaren A into allocholanolic acid.

Methyl alcoholic hydrochloric acid splits off the sugar residue and the tertiary hydroxyl group at C₁₄ from scillaren A (I), resulting in the formation of two new double bonds, Δ³ and Δ¹⁴, and the production of anhydro-scillaridin A (II). On catalytic hydrogenation, all the double bonds of this substance are reduced and, at the same time, the lactone ring is opened by reduction so that a saturated desoxycarboxylic acid is formed. This has been shown to be identical with allocholanolic acid (III) obtained by the method of Windaus⁶ and Wieland⁷ from hyodeoxycholic acid. Hence in the light of the already known constitution of allocholanolic acid, direct conclusions may be drawn regarding the constitution of the aglycone obtained from the squill glycoside, since the sequence of the reactions was such as to avoid any severe attack upon the molecule, particularly any alteration in the structure of the carbon skeleton. The close relationship between the two classes of compounds, the aglycones of the cardiac glycosides on the one hand and the bile acids on the other, is thus demonstrated in a very simple manner. That the unsaturated lactone ring in scillaren A must be 6-membered, is proved beyond doubt



Apart from D-glucose, they are all desoxysugars, i.e., they contain 1 or 2 oxygen atoms fewer than the corresponding carbohydrate with 6 C atoms. While D-glucose and L-rhamnose are fairly widely distributed, the remaining sugars have so far been found only as components of cardiac glycosides.

For the sake of completion, we have also given the formulæ for L-oleandrose and D-diginose, although the glycosides derived from these sugars will not be discussed. The chemistry of the oleander glycosides has been known for a long time and has been described in a number of comprehensive publications. Diginose is the sugar obtained from the non-cardioactive diginin, a glycoside obtained from the leaves of *D. purpurea*, which would be out of place in this article. The constitutions and configurations of both these sugars have been confirmed by synthesis^{1,2}.

The sugar residue which, in the cardiac glycosides, may consist of a chain of up to 4 sugar molecules, is responsible for the water solubility of the glycoside on the one hand and, on the other, for its power of fixation to the heart muscle.

THE STEROID STRUCTURE OF THE AGLYCONES

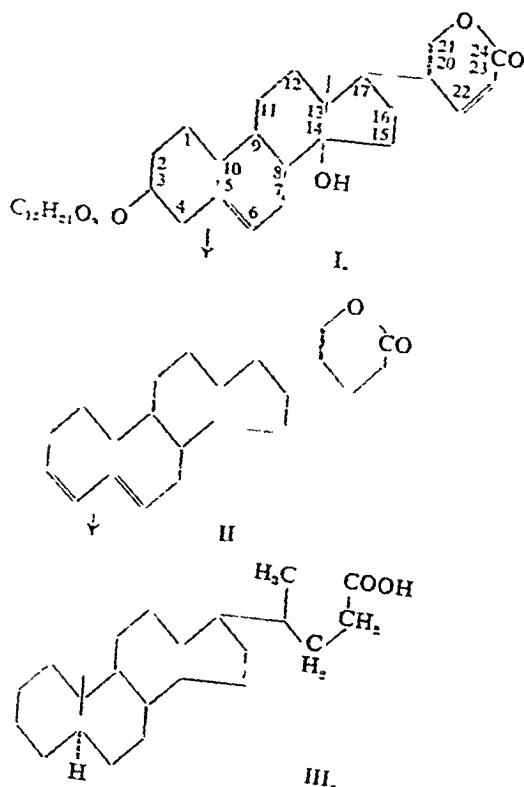
The aglycones of the cardiac glycosides possess much more complicated structures than the sugar components. These genins are the real carriers of the specific action even though, on account of their insolubility in water and their low power of fixation to the heart muscle, they are of no importance therapeutically. In structure they are very closely related to the steroids and they belong to this same large class of substances which also includes the sterols, the bile acids, the sex hormones, the hormones of the suprarenal cortex and vitamin D.

Our own investigations on the cardiac glycosides began more than 25 years ago with squill, a cardioactive drug from the Mediterranean countries which was used as a remedy for dropsy by the ancient Egyptians. Since we began our studies, we have isolated and thoroughly investigated the main glycoside of squill, scillaren A^{3,4}. Our investigations are being continued on a number of other glycosides which accompany scillaren A in squill, but which are present only in very small quantities. They are also cardioactive and some of them are very beautifully crystalline.

In connection with scillaren A, a brief description will be given of the simplest chemical conversion of this cardioactive glycoside, or of its

THE CARDIOACTIVE GLYCOSIDES

aglycone, scillaridin A, into a product of animal origin, allocholanolic acid².



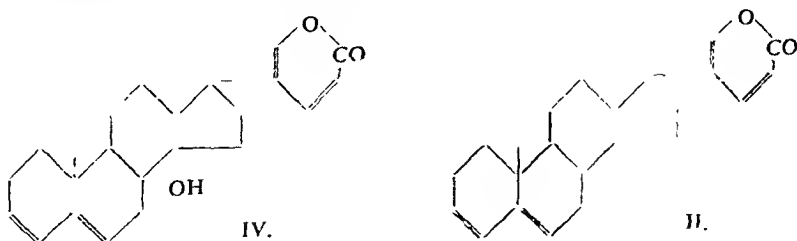
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Methyl alcoholic hydrochloric acid splits off the sugar residue and the tertiary hydroxyl group at C₁₄ from scillaren A (I), resulting in the formation of two new double bonds, Δ³ and Δ¹⁴, and the production of anhydro-scillaridin A (II). On catalytic hydrogenation, all the double bonds of this substance are reduced and, at the same time, the lactone ring is opened by reduction so that a saturated desoxycarboxylic acid is formed. This has been shown to be identical with allocholanolic acid (III) obtained by the method of Windaus⁶ and Wieland⁷ from hyodeoxycholic acid. Hence in the light of the already known constitution of allocholanolic acid, direct conclusions may be drawn regarding the sequence of the reactions obtained from the squill glycoside, since the constitution of the aglycone was such as to avoid any severe attack upon the molecule, particularly any alteration in the structure of the carbon skeleton. The close relationship between the two classes of compounds, the aglycones of the cardiac glycosides on the one hand and the bile acids on the other, is thus demonstrated in a very simple manner. That the unsaturated lactone ring in scillaren A must be 6-membered, is proved beyond doubt

by this degradation. Wieland⁸ has demonstrated that the same lactone ring is also present in the toad poisons.

In the structural formula of scillaren A, deduced from the conversion of anhydroscillaridin A into allocholanolic acid, the position of the sugar molecule and of one of the neighbouring double bonds remained uncertain. The determination of the position of the sugar residue was made more difficult by the fact that when the sugar is split off from scillaren A the hydroxyl group to which it is attached is lost at the same time and a new double bond is formed. The point of attachment of the sugar chain which is built up from rhamnose and glucose and is known as scillabiose, was at first assumed to be position 5. It was possible⁹, however, to establish with certainty that the sugar is attached to the hydroxyl group at C₃. In principle, the method was the same as that which led to the conversion of anhydroscillaridin A into allocholanolic acid, the only difference being that the sequence of the reactions was altered, the hydrolysis not being performed until after the catalytic hydrogenation so that the hydroxyl group at C₃ was retained. In this way, it was possible to show that the acid obtained from scillaren A is identical with epi-allo-lithocholic acid (3 β -hydroxy-allocholanolic acid), prepared by the method of Wieland⁷ from hyodeoxycholic acid, and, at the same time, to obtain proof that the hydroxyl group at C₃ carrying the sugar molecule bears the *cis*-configuration with respect to the methyl group at C₁₀.

In order to explain the fact that, in contrast to other rhamnosides, the sugar in scillaren A splits off easily with the formation of a conjugated system of double bonds, there must be a double bond in the neighbourhood of the hydroxyl group carrying the sugar. Hence, it follows that, by analogy with other unsaturated sterols, this ethylene linkage must be situated at $\Delta^{5,6}$. The formulæ of scillaridin A and anhydroscillaridin A corresponding to these conclusions are as follows:

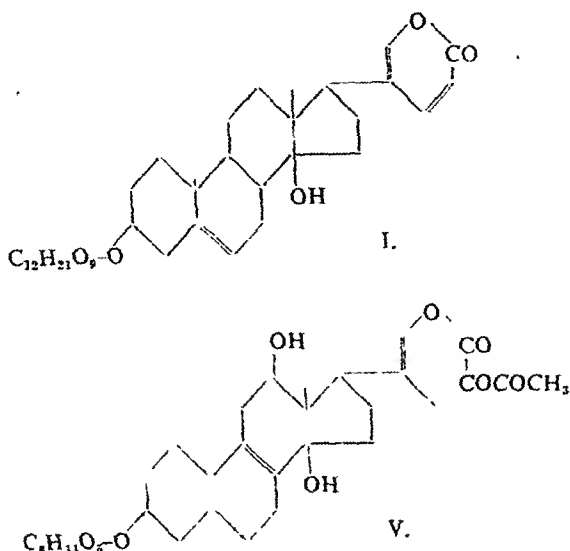


Although all the cardiac glycosides behave for the most part in a similar manner in their action on the heart, they exhibit certain differences which depend upon relatively small variations in the chemical structure. The following example illustrates how far the introduction of new groups results in changes in the type of action.

While rodents, particularly rats, are relatively speaking not very sensitive to glycosides of white squill and can tolerate comparatively large doses, they show an unusual sensitivity to one of the active principles of red squill. The red variety of squill contains a glycoside related to

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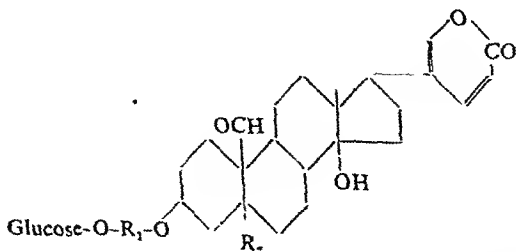
scillaren A, scillirosid, which has only been prepared in a pure crystalline state and chemically identified in the last few years^{10,11}.



Constitutional formulæ of scillaren A and scillirosid

Scillirosid, like scillaren A, has a sugar residue in position 3, although this consists only of glucose. On the other hand, it contains, in addition, a hydroxyl group, probably in position 12, as well as a double bond which is very difficult to hydrogenate and a characteristic acetyl group on the lactone ring. This acetyl group is very likely responsible for the specific toxic action in rodents. 0.1 to 0.2 mg. of scillirosid given with the food is sufficient to kill an adult rat, while the same animal could tolerate 200 times this amount of the closely related scillaren A without harm.

A glycoside with a 6-membered lactone ring has also been discovered in a species of the Ranunculaceæ. Karrer¹² isolated from the rhizome of *Helleborus niger*, the Christmas rose, a glycoside, hellebrin, with a powerful cardiac action. To this compound, he attributed the following formula VI with an aldehyde group at C₁₀ and the δ -lactone ring with two double bonds characteristic of scillaridin¹³.



VI $R_1 = \text{Glucose}$, $R_2 = \text{H}$
 VII $R_1 = \text{Rhamnose}$, $R_2 = \text{OH}$

Reichstein and his co-workers^{14,15}, who demonstrated beyond doubt the presence of the aldehyde group, suggested formula VII. They found that hellebrin was decomposed by strophanthobiasé to a monosaccharide, desglucohellebrin. On cleavage by the method of Mannich, this yields L-rhamnose and two isomeric genins, which have not yet been further investigated. In this case, too, it is possible that the sugar chain is present in the form of scillabiose.

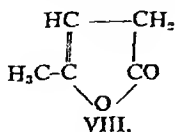
The aglycones of the cardioactive glycosides of the digitalis type and of strophanthus and certain other plants have a simple unsaturated 5-membered lactone ring in place of the 6-membered lactone ring with 2 double bonds present in scillaren A, scillirosid, hellebrin and the toad poisons. The remaining differences are connected with the peripheral structure of the molecule and depend upon the number and arrangement of the hydroxyl groups. Strophanthidin, the aglycone of k-strophanthin, like the aglycone of hellebrin, possesses the particular feature of an aldehyde group at C₁₀, whereas the other aglycones have a methyl group.

The reasoning which led to the above experimental proofs of the fine structure of the cardiac glycosides, was based upon the comprehensive knowledge which had been derived from the investigations into the steroids, and which, only about 1½ decades ago, enabled the previously very incomplete conceptions of the chemical structure of the heart glycosides to be clarified.

ATTEMPTED SYNTHESSES

That attempts to synthesise cardiac glycosides would soon follow and would lead to some interesting partial successes was to be expected. Thus Elderfield¹⁶ and his co-workers, on the one hand, and Ruzicka¹⁷ and co-workers on the other, starting from simple sterol derivatives and employing the Reformatsky reaction with bromoacetic ester, succeeded in building up the 5-membered lactone ring characteristic of many aglycones. These experiments also showed that the previous assumption of a double bond in the β,γ-position to the carbonyl group of the lactone ring was incorrect and had to be replaced by a formulation in the α,β-position.

In all the structural formulæ given here, this alteration is already taken into account. The lactone ring was assumed by Jacobs and his co-workers to be β,γ-unsaturated, i.e., aldoenol lactone, since this position of the double bond appeared to agree particularly well with its reactions, and because, in particular, this structure was claimed to give the best



explanation for the colour reaction with sodium nitroprusside, the so-called Legal test¹⁸. It had, however, already been observed at that time that, on catalytic hydrogenation, the aglycones were not reduced to saturated deoxycarboxylic acids like other enol lactones, e.g., α-angelica-

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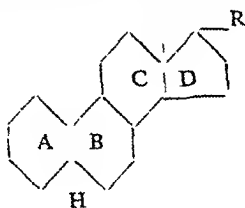
lactone¹⁹ (VIII) or scillaren A²⁰, but that they took up only two atoms of hydrogen and that the double bond was reduced without opening of the lactone ring.

Moreover the aglycones with 5-membered lactone rings do not add on bromine on titration by Winkler's method¹⁸. That all aglycones so far investigated do, in fact, contain an α,β -unsaturated lactone ring, was shown by a thorough comparison with simple synthetic lactones and with lactones derived from steroids in which the position of the double bond had been established beyond question¹⁶. These comparisons concerned principally the behaviour of the lactone ring on cleavage with aqueous and with alcoholic alkali, the ultra-violet absorption and the colour reaction with potassium ferricyanide. The synthetic steroid lactones so far obtainable do not contain all the structural features of the natural cardiac aglycones. It was comparatively easy to prepare substances with a secondary hydroxyl group at C₃ and having the 5-membered lactone ring at C₁₇ in the correct position. The introduction of the tertiary hydroxyl group at C₁₄ has likewise been successfully achieved²¹. The synthesis of compounds which possess the configuration of the natural substances, both at C₁₄ and at C₁₇, is being worked out.

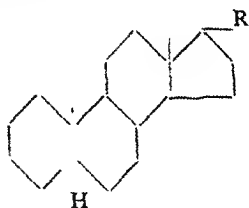
THE CONFIGURATION OF THE AGLYCONES

This brings us to a further problem, the solution of which has received particular attention in the last few years, namely the configuration of the individual linkages in the aglycones. By precise evaluation of X-ray photographs, Bernal and Crowfoot²² have shown that the natural steroids must possess a flat, relatively elongated molecule.

This necessitates that rings B and C should be united in the trans-configuration, both in the cholanic (IX) and in the allocholanic (X) series, which differ from one another in the configuration of C₅.



IX.

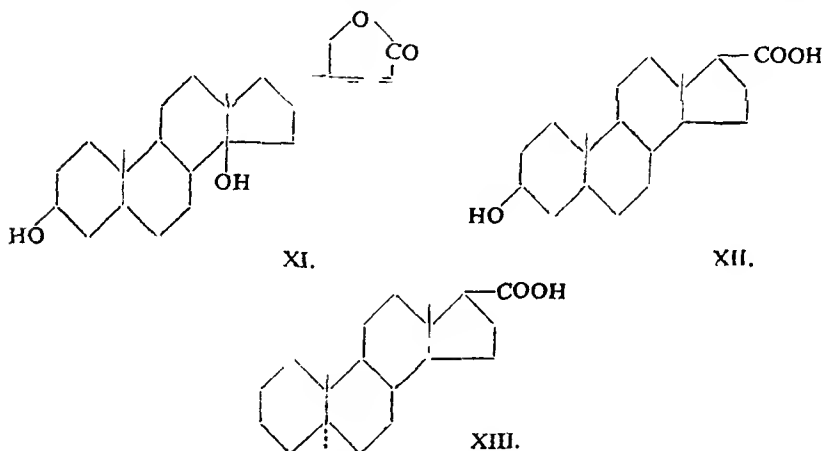


X.

The allocation of the aglycones of the cardiac glycosides to the cholanic series follows from the degradation of, for example, digitoxigenin to ætiocholanic acid. This degradation, as well as the numerous conversions which elucidated the connections between the individual aglycones, shows that the configuration of the asymmetric C-atoms 8, 9, 10, 13 and 17 in all these substances is the same as that present in the bile acids. We shall, therefore, discuss the steric relationships for those positions where particular isomerisms can occur. The two possible positions for substituents on asymmetric carbon atoms in the steroid skeleton will be denoted by the Greek letters α or β and indicated in the usual manner by dotted or by continuous valency lines. As point of reference, the C-atom in

position 10 will be selected and substituents which bear a *cis*-configuration with respect to the methyl group at this point will be defined as β -orientated.

The proof that epimerism occurs at C_3 based on the fact that cholesterol, for example, is precipitated by digitonin while certain derivatives are not, has been known for a long time. It has recently been shown, however, that not all 3β -hydroxysteroids are precipitated by digitonin, but that the precipitation can be prevented by certain substituents at other positions in the molecule. Thus, the aglycones of the cardioactive glycosides are themselves not precipitated by digitonin, and hence the configuration at C_3 had to be proved by chemical degradation in the manner shown above for scillaren A⁹. Hunziker and Reichstein²³ obtained epi- α -tiolichoic acid (3β -hydroxy α -tiolicholic acid, XII) from digitoxigenin (XI). The β -configuration of the 3-hydroxyl group



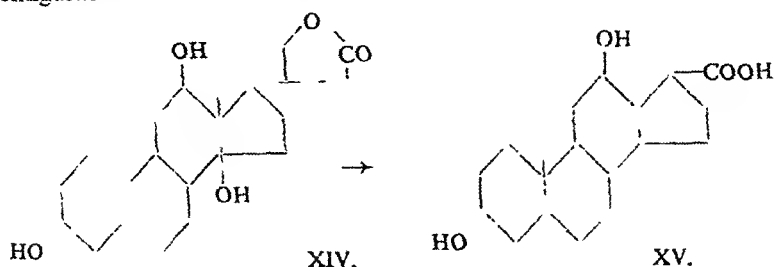
is also found in sarmentogenin²⁴ and in gitoxigenin, which has been shown, by a series of reactions which do not affect the configuration at C_3 to be related to digitoxigenin²⁵. Uzarigenin²⁶ likewise possesses a β -orientated 3-hydroxyl group, since its anhydro derivative gives a precipitate with digitonin. The opposite configuration at C_3 is found in digoxigenin²⁷ as has been shown by degradation to the corresponding 12-epi- α -tiodeoxycholic acid.

Strophanthidin and periplogenin must possess the same configuration at all asymmetric centres, since they can be transformed into identical derivatives by methods which do not affect the steric structure²⁸. The hydroxyl groups at C_3 and C_5 must bear a *cis*-configuration to one another, because they can be esterified by thionyl chloride with the formation of a neutral sulphite²⁹. The β -configuration of the two hydroxyl groups follows from the ring closure between the aldehyde group at C_{10} and the 3-hydroxyl group in the anhydro-strophanthidin derivatives³⁰, and from the fact that, if a carboxyl is added to the aldehyde group by cyanhydrin synthesis, lactone formation takes place with the 5-hydroxyl group³¹. The substituent at C_5 therefore has the same configuration as

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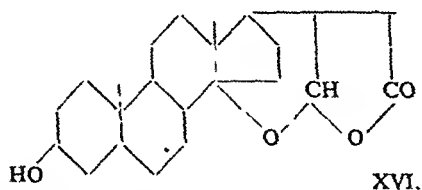
the hydrogen atom in the other aglycones, and hence strophanthidin and periplogenin also belong to the cholanic series. Only uzarigenin possesses the allocholan configuration, since it is degraded to ætio-allocholan acid (XIII)³².

The above-mentioned degradation of digoxigenin (XIV) to 12-epi-ætiodeoxycholic acid (XV) proves that the 12-hydroxyl group has the β -configuration.



In scillirosid, the configuration at C_{12} must be the same as in digoxigenin since, in certain derivatives of *iso*-scillirosid, the presence of an oxide ring between C_{21} and C_{12} can be demonstrated, which would necessitate that the side-chain and the 12-hydroxyl group should possess the *cis*-configuration³³.

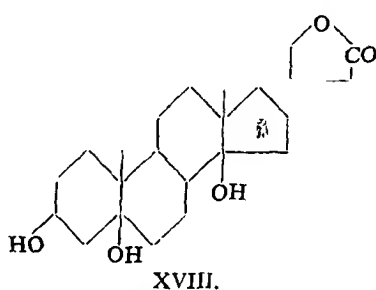
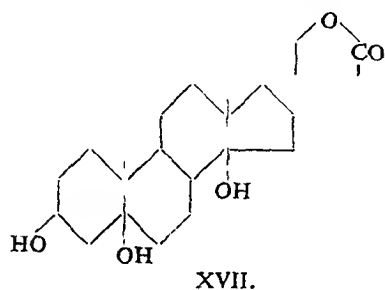
The 14-hydroxyl group which is common to all the aglycones of the cardiac glycosides must be formulated as β -orientated, since the oxide ring between C_{14} and C_{21} in the *isocompounds* of the aglycones can only be formed when the hydroxyl groups and the lactone side-chains possess the *cis*-configuration.



That the isomerisation of the aglycones by alkalis takes place without change of configuration at C_{12} , is proved by the degradation of digitoxigenin via *iso*-digitoxigenin (XVI) to ætiocholanic acid³⁴ and by degradation of anhydro-uzarigenin to allo-ætiocholanic acid³². Both acids possess β -orientated carboxyl groups. It may be mentioned here that Reichstein and his co-workers have demonstrated the β -configuration of the 14-hydroxyl groups in periplogenin³⁵ and in digitoxigenin³⁶ by another method, namely, by oxidative degradation to a 20-keto-21 \rightarrow 14-lactone. Hence, in the cardiac aglycones, rings C and D are joined in the *cis*-position, in contrast to the bile acids and the steroids. For the *cis*-union of rings C and D Reichstein³⁷ has proposed the term 14-*iso*, Ruzicka the term 14-*allo*³⁸.

Under the influence of one of the enzymes found in strophanthus seeds, the glycoside cymarín undergoes a peculiar rearrangement³⁹, resulting in the formation of an isomeric physiologically inactive glycoside which

Jacobs and his co-workers have named allocymarin. On hydrolysis with acids, this yields allo-strophanthidin which is not isomerised by alkalis, but possesses the same functional groups as strophanthidin and must, therefore, be stereoisomeric with it. The systematic removal of the asymmetry at the oxygen-substituted asymmetric centres does not lead to identical derivatives from strophanthidin and allo-strophanthidin⁴⁰. It must, therefore, be assumed that a change of configuration at C₁₇ occurs on allomerisation. Thus, the side-chain takes up a *trans*-configuration with respect to the 14-hydroxyl group and isomerisation with the formation of an oxide ring can no longer take place. Periplocymarin (periplogenin-cymarosid) can also be allomerised by means of enzymes⁴¹. Reichstein³⁵ has shown that alloperiplogenin (XVIII) differs from periplogenin (XVII) only in the configuration at C₁₇, i.e., the allo-linkage carries an α -orientated side-chain. This may also be assumed to be the case for allo-strophanthidin.



In the last few years, the introduction of simple sugars into natural aglycones and into synthetically prepared steroid lactones has been successfully accomplished, so that a whole series of partially synthetic glycosides are now available^{42,43,44,45}. In addition, it has been shown that, in pharmacological experiments, synthetic substances obtained from natural aglycones by introduction of sugar residues often exhibit a high activity which, in certain cases, exceeds that of the natural glycosides. Up till now, however, no clinical results are available relating to the therapeutic activity and utility of these compounds. In the case of substances with synthetic lactone rings, the activity, although detectable, was only very slight. They lack certain structural features necessary for a high activity, such as, above all, certain steric relationships. This would almost seem to justify the old contention that Nature, in many cases, provides the physician with the remedy in its optimum form. In this connection, the extremely favourable properties of penicillin may be recalled.

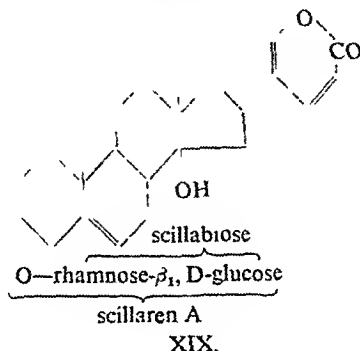
THE SUGAR COMPONENTS OF THE GENUINE GLYCOSIDES

The close relationship which the various cardiac glycosides bear to one another is also manifest with regard to the position and composition of the sugar residue. In all the cardiac glycosides so far examined, this occupies the 3-position, where the aglycone is usually united with a

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desoxy-sugar. This, in some cases, may in turn be combined with further desoxy-sugars and finally with 1 to 2 molecules of glucose. The glucose is readily split off by enzyme action and, for this reason, as already mentioned, the previously isolated glycosides such as digitoxin and gitoxin, or even digoxin from *Digitalis lanata*, lacked the terminal glucose, whereas, in the genuine glycosides obtained by excluding enzyme action, the glucose is present. The simplest example of a genuine cardiac glycoside is once more provided by scillaren A the composition of which is depicted schematically in the following diagram:

SCILLAREN A



The sugar residue is situated at C₃ and is composed of rhamnose and glucose. Acids break the linkage with the aglycone and split off scillabiose^{3,4}, while scillarenase, an enzyme accompanying the glycoside in squill, breaks the linkage between rhamnose and glucose⁴⁵. Thus, by means of this enzyme, and only in this way, a step-wise degradation may be carried out and the intermediate product proscillaridin A, a beautifully crystalline glycoside, obtained. Proscillaridin A has too low a solubility for therapeutic purposes, the only sugar present being rhamnose.

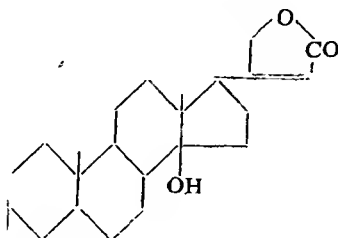
In the case of the genuine cardiac glycosides of the digitalis type, the conditions are somewhat more complicated. We had already learnt in our experiments with squill to prevent the action of the enzyme responsible for splitting off glucose. When we applied this process to the extracts of the glycosides of *Digitalis purpurea*, we obtained, instead of the already well-known crystalline glycosides digitoxin and gitoxin, amorphous but likewise very active products. The failure to crystallise was a great obstacle to the complete purification and identification of the genuine glycosides of *D. purpurea*. Only after we had first obtained experience with the glycosides of *D. lanata*²⁷ were we able to complete the investigations of the purpurea glycosides⁴⁸. From *D. lanata*, by a process excluding enzymatic action, it was possible to obtain a crystalline glycosidal preparation which soon proved to be a mixture of 3 very different isomorphous glycosides which we designated digilanid A, digilanid B and digilanid C.

The marked differences in the distribution of the individual components between chloroform and aqueous methyl alcohol enabled the total

digilanid preparation to be resolved into the homogeneous components by repeated systematic partition between the two solvents. Separation of the components by fractional crystallisation is rendered very difficult by the isomorphous nature of the crystals.

The space at our disposal is not sufficient for a detailed description of the three digilanids A, B and C, and the products of their step-wise degradation. We shall, therefore, confine ourselves to digilanid A. Analogous conditions exist for digilanids B and C. The sugar chain is identical in all three digilanids.

SCHEME FOR DIGILANID A



O-digitoxose-digitoxose-acetyldigitoxose- β , D-glucose
XX.

The formula shows the unsaturated lactone ring in position 17 and 2 hydroxyl groups in positions 3 and 14, the one in position 3 carrying the sugar chain. The latter consists of 3 molecules of digitoxose and a terminal glucose. The third molecule of digitoxose also carries an acetyl group which is characteristic for the digilanids and is responsible for the isomorphism. If it is removed, the A and B components lose their power of crystallisation⁴⁹. They yield amorphous substances which have proved to be identical with the likewise amorphous genuine glycoside of *D. purpurea*.⁴⁸ These, in turn, by enzymatic removal of the terminal glucose, are converted into digitoxin or gitoxin. Thus the relationship between the glycosides of *D. lanata* and *D. purpurea* is clearly shown. The difference between the genuine glycosides of the two plants consists therefore in the presence of an acetyl group in the lanata glycosides.

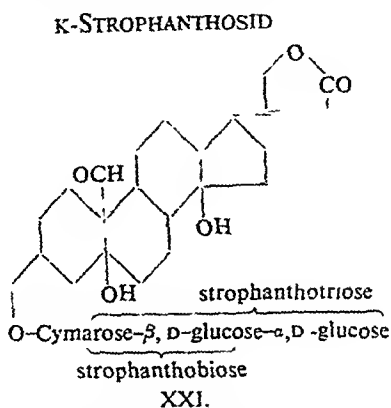
For digilanid C, which has a composition very similar to that of digilanid A, there is no corresponding glycoside in *D. purpurea*. The digilanid C-structure has so far been found only in *D. lanata* and, as Mannich⁵⁰ has shown, in *D. orientalis*, a variety very similar to lanata which is found in Asia Minor.

The best example of the step-wise degradation of a genuine glycoside is provided by strophanthin or k-strophanthosid as we designate the crystalline active principle of the seeds of *Strophanthus kombé*. Jacobs⁵¹ in New York had already isolated in small quantities from this drug two crystalline glycosides: cymarín, consisting of the aglycone strophanthidin and cymarose, and k-strophanthin- β , which contains one glucose molecule more than cymarín. The main part of the glycosides

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isolated from this drug remained amorphous but, in the form of strophanthin, a greatly purified preparation, they have been widely used for many years. In this connection, the pioneer work of Fraenkel⁵² deserves especial mention.

By employing a special process utilising the peracetyl derivative (heptacetyl) we have succeeded in converting the main portion of the total glycosidal preparation from strophanthus seeds into a crystalline and pure form and have introduced it into therapy as k-strophanthosid, known commercially as "Strophosid"⁵³. The following scheme shows clearly the composition and the step-wise degradation by which all the cleavage products could be isolated and characterised.

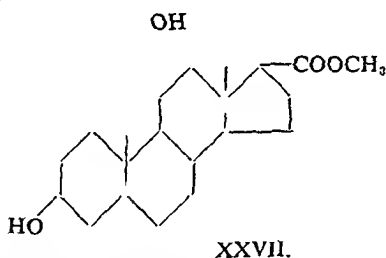


The aglycone, strophanthidin, possesses hydroxyl groups at C₃, C₅ and C₁₄. At C₁₇ the 5-membered unsaturated lactone ring is attached and at C₁₀ the aldehyde group characteristic of strophanthidin. As in the case of the other cardioactive glycosides of the digitalis group, the oxygen atom at C₃ forms the bridge to the sugar chain, which, in k-strophanthosid, consists of cymarose and two molecules of glucose. If the terminal glucose is split off by enzymatic hydrolysis, k-strophanthin-β is produced. By splitting off a further molecule of glucose with the specific enzyme strophanthobiase, k-strophanthin-β is converted into cymaridin which consists only of the aglycone strophanthidin and cymarose. The linkage between the aglycone and the sugar chain is broken by acid. From k-strophanthosid, a sugar, strophanthotriose, is obtained which consists of one molecule of cymarose, and two molecules of glucose. Cleavage of k-strophanthin-β leads to strophanthobiose, composed of cymarose and glucose. Acid cleavage of cymaridin yields the aglycone and cymarose.

The example of k-strophanthosid enables an exceptionally good insight to be obtained into the fine structure of the glycosidal linkages and, hence, into the nature of the enzymes which are responsible for sugar cleavage and are specific for the cardioactive glycosides. As the nature of these enzymes was originally unknown, nothing could be said regarding the configuration of the linkages between the sugars. It was found that a well-known enzyme, the α-glucosidase of yeast, was capable of splitting the linkage between the two glucose residues of k-strophanthosid, so that,

those involved in the glycosidal linkages. This argues against a tertiary hydroxyl group at C₅, unless it is assumed that, in ouabain, in contrast to other cardiac glycosides, the sugar is attached at this point. By a number of ring closures, Mannich has provided good evidence for the positions of the other hydroxyl groups. As regards the configuration of the asymmetric C-atoms to which the oxygens are attached, it can only be said that all the hydroxyl groups are *cis*-orientated to one another.

The seeds of *Strophanthus sarmentosus* contain a mixture of glycosides none of which has yet been prepared in its native state, although three glycosides, sarmentocymarin⁶¹ and sarmentosids A and B⁶² have so far been obtained after enzymatic removal of glucose. Sarmentocymarin, an already well-known glycoside, yields, on acid hydrolysis, sarmentogenin and the methyl ether of a 2-desoxy-methylpentose, sarmentose. Although the configuration of this sugar has not yet been established with absolute certainty, on page 850 we have assigned to it the configuration of 2-desoxy-D-idomethylose (2-desoxy-D-gulomethylose). On the assumption that sarmentose possesses a straight C-chain, this is the only possible configuration, since, from each of three of the four pairs of isomers theoretically possible, at least one partner is known and sarmentose is neither identical nor enantiomorphous with any of these. The structure of sarmentogenin has been established by degradation to 3 β -11 α -diacetoxy- α -tiocholanic acid methyl ester²⁴ as that depicted in formula XXVII.



In the case of sarmentosids A and B only the sugar components are known. Sarmentosid A contains L-talomethylose⁶³, a sugar which had not previously been found in a natural product. Sarmentosid B contains the sugars D-glucose and digitalose⁶². A striking feature here is that the glucosidal linkage is resistant to the attack of the specific enzyme.

CARDIAC GLYCOSIDES OF UNOFFICIAL DRUGS

Of the remaining drugs which contain cardiac glycosides, only those whose active principles have been chemically investigated during the last 10 years will be discussed. First of all, mention should be made of *Convallaria majalis*. Convallatoxin, the glycoside contained in its leaves and flowers, was isolated and described a long time ago by W. Karrer⁶⁴. Reichstein and Katz⁶⁵ were, however, the first to elucidate its constitution, using the method of Mannich which hydrolysed it to strophanthidin and L-rhamnose. The seeds of *C. majalis* also contain cardiac glycosides. One of these was isolated in a pure state by Schmutz

and Reichstein⁶⁶ and given the name convalliosid. It is split by strophanthobiase to yield convallatoxin and D-glucose. Thus, its sugar structure corresponds to that of scillaren A. Nevertheless, it has not yet been possible to split off the sugar chain as a whole, but this would presumably be in the form of scillabiose. Convalliosid is very probably the genuine glycoside of the seeds of *C. majalis*.

The roots of *Adenium somalense*, a plant of the Family Apocynaceae, together with varieties of strophanthus and acocanthera, are used by certain native African tribes, particularly in Kenya, for the preparation of arrow poisons. It has been found that they contain a crystalline glycoside to which Hartmann and Schlittler⁶⁷ have given the name somalin. On acid hydrolysis, somalin is decomposed into digitoxigenin and cymarose and is therefore so simple in structure that, apart from the configuration of the glycosidal linkage, all the structural details can be deduced merely by hydrolytic cleavage.

The aglycone of the glycosides present in the nuts of *Thevetia nerifolia* has been identified as digitoxigenin⁶⁸. At the same time, it was shown that thevetin is decomposed both by the drug enzymes^{69,70} and by strophanthobiase⁷⁰ and the digestive enzymes of snails⁷¹, yielding the glucose-free nerifolin. The isolation of acetylnerifolin suggests that the genuine glycoside is present as an acetyl derivative of thevetin. Similar glycosides have been found in the nuts of *Cerbera odollam*. Of these cerberosid⁷², which is assumed to be the genuine glycoside, is converted by enzymatic degradation^{73,74} into nerifolin or monoacetylnerifolin (cerberin)^{75,76}.

Thevetose is also the characteristic desoxysugar of the glycosides contained in the nuts of *Tanghinia venenifera*, another member of the Apocynaceae⁷⁷. Frèrejacque and Hasenfratz were able, after isolating several glucose-free glycosides from the amorphous, acetyl-containing tanghiniosid, to prepare gentiobiose, so that here, too, the nature of the sugar residue has been to a large extent cleared up⁷⁸.

The leaves of *Adonis vernalis* (Ranunculaceae) contain at least two glycosides, cymarín and adonitoxin⁷⁹, which, together, may be considered to be mainly responsible for the pharmacodynamic action of this drug. Adonitoxin has been further investigated by Katz and Reichstein. It contains the sugar component L-rhamnose united with an aglycone which is isomeric, but certainly not identical with strophanthidin. This aglycone, likewise, possesses an aldehyde group and the simply unsaturated γ -lactone ring characteristic of the genins of digitalis and strophanthus glycosides, but, in contrast to strophanthidin, it has only one tertiary and two acylable hydroxyl groups.

From the seeds of *Cheiranthus cheiri* (Goldlack), a cardioactive glycoside cheirotaxin can be isolated⁸⁰. On cleavage by the method of Mannich, this yields strophanthidin and a sugar syrup from which phenyl glucosazone can be obtained. In addition to glucose, however, cheirotaxin contains a pentose, the D-lyxose⁸¹ which is the first pentose found as a component of cardiac glycosides.

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RESEARCH PAPERS

SODIUM AND CALCIUM GLYCEROPHOSPHATES

A SURVEY

BY J. S. TOAL AND J. I. PHILLIPS

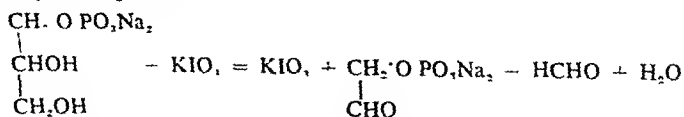
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THE object of this paper is to draw attention to the unsatisfactory position now existing in which commercial supplies of glycerophosphates, although issued as complying with the standards of the British Pharmaceutical Codex, do not actually satisfy the full requirements of the monographs. In order to clarify this position the authors of the present paper produced some pure salts, following factory practice, and investigated them. Published work by other investigators in this subject may be referred to from the bibliography given at the end of the paper^{1,2,3,4,5,6,7,8}.

DIFFERENTIATION BETWEEN THE ISOMERS

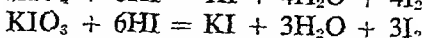
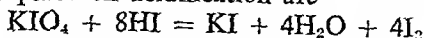
In order to differentiate between the α and β -salts of glycerophosphoric acid an efficient method of assay was required. This was found in the Malaprade reaction of periodic acid on vic. glycols for the estimation of the α -isomer. This method was first applied by Fleury and Paris⁹ to the assay of α -glycerophosphates, using arsenious acid for determining the excess of periodic acid, and was later adopted by Pyman and Stevenson⁶. But the present authors found the arsenious acid method somewhat difficult to manipulate, and they prefer the direct procedure of acidifying in the presence of excess of potassium iodide and determining the amount of iodine lost in the reduction of the periodic acid to iodic acid, in comparison with a standard blank. Both methods gave results in complete agreement.

0.3 g. of the crystalline sodium glycerophosphate (or 0.15g. if the α content exceeds 50 per cent.) is weighed, placed in a stoppered 250-ml. conical flask and dissolved in a minimum amount of water; 25 ml. of a periodic acid solution (prepared by dissolving 3.674 g. of $\text{Na}_3\text{H}_2\text{IO}_6$ in 37.5 ml. of N sulphuric acid and diluting to 500 ml.) is added, the flask is swirled and set aside for 10 minutes. The reaction that takes place is represented by the equation—



After standing, 1 g. of sodium bicarbonate is added to the flask followed by 5 g. of potassium iodide and 10 ml. of dilute hydrochloric acid. The carbon dioxide evolved is allowed to displace the air, the flask is stoppered, and set aside for 10 minutes, and the solution finally titrated with 0.1N sodium thiosulphate. A blank must be carried out in a

similar manner and the difference between the two titrations noted. The reactions that take place on acidification are—



Thus when 1 molecule of potassium periodate is reduced to potassium iodate a loss of iodine is shown in the final titration.

Hence, from the first equation above, 1 gram-molecule of sodium glycerophosphate is equivalent to 1 gram-molecule of potassium periodate, or 1 gram-molecule of iodine, or 2 litres N/1 thiosulphate. Thus each ml. of 0.1N thiosulphate in the difference noted is equivalent to 0.0162 g. of the α -salt $\text{C}_3\text{H}_7\text{O}_6\text{PNa}_2 \cdot 6\text{H}_2\text{O}$.

In a mixture of the isomers, the amount of α -sodium glycerophosphate can be found by the above method, and the amount of the β -salt determined by titrating the total glycerophosphate and deducting from that result the titre due to the α -salt. The indicator in the titration should be methyl yellow, which gives a much sharper end point than methyl orange. At this stage of the work it had been assumed that the samples crystallising from the liquors were pure mixtures of α and β -isomers and contained no other titratable matter.

Samples of calcium glycerophosphate were assayed in a similar manner, taking 0.125 g. of the salt, dissolving this directly in 25 ml. of the periodic solution and completing the titration in the manner already described; the difference between the titration of the blank and the test being recorded, 1 ml. of 0.1N thiosulphate being equivalent to 0.0105 g. of anhydrous calcium glycerophosphate.

When analysing sodium glycerophosphate liquors from the original combination, and also the mother liquors from which crops of crystals have been taken, it is essential that any free glycerin shall be completely removed, by repeated extraction with alcohol, because even traces of glycerin give rise to error in the assay for the α -salt.

PREPARATION OF PURE α AND β -SODIUM GLYCEROPHOSPHATE

Crude sodium glycerophosphate was prepared by combining, under vacuum, two equivalents of glycerin with one equivalent of sodium acid phosphate and then hydrolysing the resultant diglyceryl ester with sodium hydroxide. The glycerin was removed by extraction with alcohol and, after separation, the alcohol that remained was expelled by evaporation. Samples from three bulk batches were adjusted to contain 50 per cent. w/w of $\text{C}_3\text{H}_7\text{O}_6\text{PNa}_2 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ and the solution analysed. The results are shown in Table I.

TABLE I

Batch	Sp. gr. at 15.5°C.	Assay by titration	Assay by ignition	Residue at 150°C.	α -isomer (6H ₂ O)
		per cent	per cent.	per cent.	per cent
A	1.274	50.0	50.7	35.4 (theory 34.3)	23.15
B	1.277	49.5	50.6	35.4	21.0
C	1.277	50.0	50.7	36.3	22.0

These figures show the general constancy of the combination.

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The bulked solution of these three batches was evaporated and adjusted to a strength of 70 per cent. w/w of $C_3H_7O_6PNa_2 \cdot 5\frac{1}{2}H_2O$ and allowed to crystallise during several days. The crystals were removed and washed with alcohol. The mother liquor was again set to obtain further crystals and this procedure repeated until six different crops had been collected. The liquor from the sixth crop was uncrystallisable.

These six crops of crystals, after washing with alcohol to ensure the removal of any adherent glycerin, were assayed for their α β -content. The results are shown in Table II.

TABLE II

Crop	α -salt ($6H_2O$)	β -salt ($5H_2O$)	Total
	per cent.	per cent.	per cent.
1	13.9	85.2	99.1
2	71.0	30.9	101.9
3	91.5	7.85	99.35
4	28.5	72.9	101.4
5	71.0	30.1	101.1
6	42.0	57.8	99.8

Each of the six samples satisfied all the chemical requirements of the B.P.C., but all failed to comply with the opening definition that "sodium glycerophosphate is the sodium salt of β -glycerophosphoric acid."

Table II shows that the first crop of crystals contain the highest proportion of the β -isomer, whereas the third crop consisted almost entirely of the α -variety. These two crops of crystals were taken separately and twice recrystallised; finally separated from their mother liquor by centrifuging then washed with alcohol and dried at $60^\circ C$.

The analysis of these recrystallised preparations (given in Table III) shows that two pure isomers were obtained, the α -isomer containing 6 molecules of water of hydration and the β -isomer 5 molecules.

TABLE III

Tests	1st crop (twice recrystallised) β -isomer	3rd crop (twice recrystallised) α -isomer
Loss at $150^\circ C$.	per cent. 29.75 (theory 29.42)	per cent. 33.25 (theory 33.34)
Assay (titration)	99.30 $C_3H_7O_6PNa_2 \cdot 5H_2O$	99.50 $C_3H_7O_6PNa_2 \cdot 6H_2O$
Assay (gravimetric)	99.20	99.20
α -isomer	absent	99.00
Sp. gr. of 50 per cent. w/w solution	—	1.271 at $15^\circ 5' C$.

A saturated solution of the α -isomer contained the equivalent of 63.5 per cent. w/w of $C_3H_7O_6PNa_2 \cdot 6H_2O$ and a saturated solution of the β -isomer contained 45.5 per cent. w/w of $C_3H_7O_6PNa_2 \cdot 5H_2O$.

The crystals of the α -isomer were hard and of a semitransparent nature, quite distinct from those of the β -isomer, which were fragile and of needle-like appearance.

The uncrystallisable liquor, which represented 10 per cent. of the original combination, was diluted with sufficient water to keep any α and β -calcium glycerophosphate in solution and then treated with a solution of calcium acetate. The precipitate that formed was collected

and analysis indicated its being a di-ester $\text{CHOH}(\text{CH}_2\text{OPO}_3\text{Ca})_2$; the figures based on material dried at 150°C . are given in Table IV.

This calcium salt did not reduce periodic acid solution, and therefore was not a vic. glycol. An approximate computation showed that 100 g. of presumptive sodium glycerophosphate in the uncrystallisable mother

TABLE IV

	Calcium	Residue on ignition	Phosphorus
	per cent.	per cent.	per cent.
For suggested formula	24.4	77.5	18.90
For the precipitate	25.5	78.1	18.60

liquor consisted of 55 parts of di-ester and 45 parts of true sodium glycerophosphate; yet, when adjusted to a liquor containing 50 per cent. of apparent sodium glycerophosphate ($5\frac{1}{2}\text{H}_2\text{O}$) as estimated by titration, it agreed with the standards set in the B.P.C.

CALCIUM GLYCEROPHOSPHATE

Calcium salts were prepared from solutions of the pure α and β -salts of sodium glycerophosphate.

With the β -salt, the method employed was that of treating the solution with an excess of 30 per cent. w/v aqueous solution of calcium acetate, and following with an excess of alcohol sufficient to throw down the calcium glycerophosphate. This was collected, washed free from calcium and sodium acetates by means of alcohol, dried and then examined. It held one molecule of water of hydration ($1\text{H}_2\text{O}$) and was soluble to the extent of 0.96 g. of anhydrous material in 100 ml. of solution at room temperature.

With the α -salt, two methods of preparation were employed: one precisely as just described for the β -salt; the other, that of using more concentrated solutions (without the use of alcohol) and allowing the calcium glycerophosphate to crystallise from the supersaturated condition. In both cases the material was collected, washed with alcohol, dried and analysed. The precipitate effected by means of alcohol contained 2 molecules of water of hydration ($2\text{H}_2\text{O}$), and that depositing from the supersaturated solution only 1 molecule ($1\text{H}_2\text{O}$). The analytical figures for the 3 salts are given in Table V.

SOLUBILITY OF THE TWO α -SALTS IN WATER

Each salt was treated with distilled water, leaving a little of the salt undissolved. After about an hour's digestion 50 ml. of solution was withdrawn and titrated and the amount of calcium glycerophosphate in solution calculated. The solution of the $2\text{H}_2\text{O}$ salt contained 4.3 per cent. of the anhydrous salt and that of the $1\text{H}_2\text{O}$ salt 1.17 per cent. The suspensions were set aside for 3 days, with occasional shaking, and then again tested. The amount of $2\text{H}_2\text{O}$ salt in solution had dropped to 4.0 per cent., whilst that of the $1\text{H}_2\text{O}$ salt remained at 1.17 per cent. The

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mixture containing the $2\text{H}_2\text{O}$ salt was now tested periodically until constant solubility was attained. This occurred at 1.26 per cent. of the anhydrous material after about 4 weeks' digestion at from 20° to $25^\circ\text{C}.$; considerable precipitation having taken place. The residuum of this digest was collected, washed with alcohol, dried and examined: it consisted of α -calcium glycerophosphate now associated with only 1 molecule of water of hydration. Thus a change in constitution has occurred, and the more soluble but unstable salt with 2 molecules of water has reverted to the less soluble but stable salt with 1 molecule of water.

These 2 α -hydrates were rendered anhydrous: the $2\text{H}_2\text{O}$ salt lost all the water at $140^\circ\text{C}.$, but the $1\text{H}_2\text{O}$ salt had to be dried at $150^\circ\text{C}.$ On exposing the anhydrous salts to the atmosphere, both absorbed moisture and returned to their original states of hydration, that is to say, to the $2\text{H}_2\text{O}$ and $1\text{H}_2\text{O}$ hydrates, and dissolved 1 in 25 and 1 in 100 of water respectively, just as they did before subjection to dehydration. Thus there are 2 hydrates chemically the same, but having different solubilities. Possibly, the structures of the two hydrates differ, and a different arrangement of the calcium and glycerophosphate ions and of the molecules of water in the lattices could explain the effects noted.

X-ray diffraction data of the two hydrates and of the corresponding dehydrated salts are given below.

TABLE V

X-RAY DIFFRACTION DATA OF THE TWO HYDRATES OF α -CALCIUM GLYCEROPHOSPHATE, INTERPLANAR SPACINGS IN Å WITH APPROXIMATE RELATIVE INTENSITIES

Monohydrate	Dehydrated Monohydrate	Dihydrate	Dehydrated Dihydrate
13 0 vs 6 7 w. about 4 0 w. diffuse band	14 8 vs about 4 0 s. diffuse band	14 2 vs. 7 1 mw	10 9 vs broad line 4.19 mw
5 5 mw 4 64 s. 3 34 mw 2 89 m 2 75 w 2 33 w 2 10 vw 1 83 w	2 73 w 1 99 w	5 17 m 3.49 m 2 22 vw 2.05 w. 1.81 w 1 72 w	3 86 ms. 3.48 ms 3.27 w. 3.04 mw 2.57 w. broad 2 11 w. broad 1.85 vw. 1 73 vw.

s Strong m Medium w Weak.

These experimental data demonstrate the distinct structural difference of the two hydrates and of their respective dehydrated salts. It is interesting to compare the effect of dehydration on the diffraction patterns, and in particular on the strong high spacing lines, in the 2 cases. For the dehydrated monohydrate the pattern is almost non-existent except for a strong diffuse band and an extremely strong line of higher spacing than the maximum spacing line of the original monohydrate. This suggests that the water molecules are important in holding the calcium glycerophosphate units of the structure together, and that on their removal these units drift apart and the structure becomes much less ordered. On the other hand, the dehydrated dihydrate has a good strong pattern with a decrease in spacing of the maximum spacing line from that of the original dihydrate. This suggests that on removal of the water mole-

cules the remaining calcium glycerophosphate units can pack together in a highly ordered crystal structure which can, however, readily revert to that of the original hydrate.

COMMERCIAL SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Samples of this salt were obtained from the principal manufacturers and subjected to the B.P.C. tests: the results are given in Table IV. The last three samples in the table were prepared by the present authors.

TABLE VI
ANALYTICAL DATA OF SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Sample	Loss at 150°C.	Assay by titration	Assay by ignition	α -isomer	Solution 1 in 50
	per cent.	per cent.	per cent.	per cent.	
A ...	7.6	93.0	99.46	79.76	Soluble; but flocculent. Precipitate overnight.
B ...	10.8	94.60	98.80	78.92	Soluble but within 1 hour commenced to flocculate.
C ...	7.5	94.18	101.16	79.64	Not complete; also commenced to flocculate on standing.
D ...	10.5	93.90	97.85	51.91	Soluble; flocculated on standing.
α -salt 2H ₂ O ...	13.9	98.90	99.63	99.15	Soluble; only very slight flocculation overnight.
α -salt 1H ₂ O ...	7.0	99.33	99.5	100.90	Not complete but dissolves quickly 1 in 120 to a clear solution.
β -salt 1H ₂ O ...	9.8	100.22	100.0	absent	Not complete but dissolves slowly 1 in 120.
B.P.C. Standards	Not more than 15.0	94.50	98.0	—	Dissolves with slight turbidity.

All figures are based on material dried at 150°C.; B.P.C. directs drying at 130°C.

Reference to Table VI will show that 3 out of the 4 samples analysed failed to meet the standard set for titratable matter, and yet reached the requirement (or practically so) as regards ash. This at once brings up the question as to whether the preparations or the standards are wrong; and when the different solubilities are also taken into account, emphasis is added to the question. It is clear that there are some difficulties in manufacture, for otherwise there would be greater uniformity in the products, and it may be asked whether the requirements of medicine are such that more precise standards, to secure a closer approach to the pure salt, should be set up or whether such differentiation is of little therapeutic importance and wider limits should be allowed: such wider limits being, of course, rather arbitrary and merely arranged to suit good commercial practice. But on the question of solubility there is room for debate: insisting upon a degree of solubility which is not a stable property of the salt serves no useful purpose, but rather the reverse. If a pharmacist looks to a salt being soluble 1 in 50 of water and then, when using it in compounding, finds precipitation takes place, he is perturbed and at a loss; but if he knows that an ultimately stable solution can only exist at 1 in 100 some of his difficulties will not arise. Again, if there is no sound therapeutic reason for preferring one or other of the isomers, then degree of solubility in water is of still less importance since both salts will be readily soluble in the acid gastric juice.

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PROPOSED CHANGES TO THE B.P.C. MONOGRAPHS FOR SODIUM AND
CALCIUM GLYCEROPHOSPHATE

Resulting from their present investigation the authors suggest that the following changes might be made in the B.P.C. monographs.

Sodium Glycerophosphate

1. To omit the chemical formula and also the description referring to the material as "the sodium salt of β -glycerophosphoric acid." Instead, the description to be "Sodium glycerophosphate may be the sodium salt of α -glycerophosphoric acid $C_3H_7O_6PNa_2 \cdot 6H_2O$ or the sodium salt of β -glycerophosphoric acid $C_3H_7O_6PNa_2 \cdot 5H_2O$ or any mixture of these isomers."
2. The assay to remain as at present, but referred to the material dried at $150^\circ C$. Standards to be altered to "Not less than 98 per cent. and not more than 102 per cent. of $C_3H_7O_6PNa_2$ calculated on the material dried at $150^\circ C$."
3. Loss at $150^\circ C$. not to exceed 34.5 per cent. This admits the hexahydrate with 33.34 per cent. of water and allows a little additional moisture.

Sodium Glycerophosphate 50 per cent. Liquor

1. To be described as "An aqueous solution containing about 50 per cent. w/w of α -sodium glycerophosphate hexahydrate, or a mixture of the α and β -isomers."
2. The assay to refer to the equivalent of anhydrous sodium glycerophosphate in the liquor. The limits to be 32.6 per cent. and 36.0 per cent. of $C_3H_7O_6PNa_2$.

These limiting figures are derived from the following considerations—50 per cent. of the hexahydrate is represented by 33.6 per cent. of anhydrous salt, which becomes 32.6 per cent. when allowing for a purity limit of 98 per cent. Similarly 50 per cent. of the pentahydrate is equivalent to 35.3 per cent. of anhydrous salt, and this, calculated to the upper limit of 102 per cent. allowed for the crystalline salt, becomes 36.0 per cent.

3. The specific gravity range to be from 1.255 to 1.300. This covers the two limits of the assay and also allows for the presence of 2 per cent. of glycerin.
- All the other present limits, including that for glycerin, could remain since no difficulty has been experienced in meeting these standards.

Calcium Glycerophosphate (Dihydrate)... Now included in the B.P.C.

1. The loss on drying to be determined at $150^\circ C$.
2. The assay should be the method of Bennett and Campbell¹⁰ in which igniting with ammonium nitrate and reigniting with nitric acid is adopted. The present authors found this method very satisfactory.
3. 1 g. of the salt should dissolve in 50 ml. of water at a temperature below $20^\circ C$. within a few minutes. On further dilution to 100 ml. and standing overnight no more than a very slight precipitate should develop.

The test for titratable matter and other limiting tests allow ample margin for the manufacturers' difficulties in production.

Calcium Glycerophosphate (Monohydrate)... Not recognised in the B.P.C.

1. It is recommended that official recognition be given to both α and β -salts both approximating to the monohydrates.

These are the salts which are stable in water, and which satisfy all the official requirements at present set out for the dihydrate with the single exception of solubility; the present requirement being that of an unstable condition.

1 g. of the salt should dissolve in 130 ml. of water within a few minutes. Both salts will dissolve in a smaller quantity of water, but the rate of dissolution is slow with the β -salt.

SUMMARY

1. A method has been described for the determination of the α -isomer of glycerophosphoric acid.

2. The preparation and properties of pure crystalline α - and β -sodium glycerophosphate have been described.

3. The isolation of "an impurity," occurring from a side reaction in the primary combination and which seems to approximate to a di-ester, is reported.

4. Two types of α -calcium glycerophosphate have been prepared: a dihydrate which is unstable in water and a monohydrate which is stable.

5. The properties of the two hydrates of the calcium salt have been investigated and differences in their physical behaviour are reported.

6. The preparation and properties of β -calcium glycerophosphate have been described.

7. Commercial samples of calcium glycerophosphate have been examined and their departure from official standards noted.

8. Suggestions for the official monographs have been made.

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6. Charpentier and Bocquet, *C.R. Acad. Sci., Paris*, 1932, 194, 104
7. Pyman and Stevenson, *J. chem. Soc.*, 1934, 448.
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DISCUSSION

The paper was presented by Mr. J. S. Toal.

MR. G. J. W. FERREY (Manchester) said that 23 years ago it was shown that the calcium glycerophosphate then on the market was the monohydrate, and not the dihydrate which was introduced into the Codex later. The 50 per cent. solution of sodium glycerophosphate was standardised by some makers on anhydrous sodium glycerophosphate and by others on the basis of the salt containing $5\frac{1}{2}$ molecules of water of crystallisation, while others varied between those two limits. There was very little diester in some makes. The insolubility of calcium glycerophosphate had been a difficulty in making the compound syrup. He wondered whether it would not be best to leave out the calcium glycerophosphate altogether and put in some soluble form of phosphoric acid; the main object of putting glycerophosphate in was to get the tonic effect of the phosphorus, and they could very well dispense with the calcium. He asked whether there was any difference in therapeutic activity between the α - and β -glycerophosphoric acids, and also whether there was any evidence to show that glycerophosphoric acid and its salts had any therapeutic action which was not possessed by phosphoric acid and its salts.

DR. G. E. FOSTER (Dartford) asked for information about magnesium glycerophosphate. This formerly came from Germany and usually complied with the requirements of the British Pharmaceutical Codex. After the war, however, it had been difficult to get any of British manufacture which complied.

DR. K. BULLOCK (Manchester) criticised the author's statement that the solubility of one of the isomers was not of great importance, because in any case it would dissolve in the acid gastric juice. Surely the salt would be reprecipitated again in the intestine; few substances were absorbed from the stomach. If, on the other hand, it was a real decomposition, why should not the separate products be given, instead of combining them and then allowing them to disintegrate again in the stomach?

MR. R. W. GILLHAM (Leeds) asked whether the authors considered that their work had thrown any light on the problem of the precipitation which occurred in samples of compound syrup of glycerophosphates.

MR. J. S. TOAL, in reply, referring to the calcium salt, said that one could get the dihydrate only by precipitation with alcohol. The two hydrates had different solubilities. The dihydrate would dissolve with a 1 in 20 initial solubility, and a stable solubility of 1 in 100. The monohydrate had an initial solubility of 1 in 100. They had found that 50 per cent. solutions were low in diester. He suggested that the formula for the compound syrup of glycerophosphates ought to be based on the pure salts; as it was now each manufacturer had a different preparation; and they did not comply with the B.P.C. With one or two exceptions, none of the magnesium salts on the market to-day would pass the Codex tests. The standard required 97 per cent. by ignition and 95 per cent. by titration, but many of them were less than 90 per cent.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART I. ENUMERATION AND DISTRIBUTION OF ORGANISMS IN SPRAY-DRIED POWDERS

BY KENNETH BULLOCK, WINIFRED G. KEEPE AND E. A. RAWLINS

From the Department of Pharmacy of the University of Manchester
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INTRODUCTION

THE growth and metabolism of bacteria and their response to altered environmental conditions when growing in liquid media, or such media solidified by addition of a small percentage of agar, have been extensively studied both quantitatively and qualitatively. Much has been written about the enumeration of bacteria suspended in liquids. On the contrary, the behaviour and enumeration of bacteria in powders, pastes, and oils, that is to say in systems of relatively low moisture content, have been studied to a much lesser extent. This situation is probably due to the difficulty, experienced in the past, of obtaining powders of predetermined composition, containing an even distribution of suitable numbers of a known organism, with which the experiments could be carried out.

As long ago as 1909 Shackell¹ called attention to the possibilities and advantages of freeze-drying for the purpose of obtaining bacterial cultures in a form in which they would retain their viability, cultural characteristics and state of virulence. Swift², and Elser, Thomas and Steffan³, showed that hæmolytic streptococci and meningococci were resistant to freeze-drying and subsequently remained viable over a long period of time. Heller⁴ used this method of drying for the quantitative investigation of environmental factors affecting dried samples of *Streptococcus pyogenes* and *Escherichia coli*. He did not, however, specify his limits of error. The products of freeze-drying tend to occur as flakes, and an examination of the literature has not revealed any report establishing that viable micro-organisms are uniformly distributed in the powders formed from such products.

The work described in the present communication was foreshadowed in a previous paper from this Department⁵. It was there suggested that a spray-dried powder containing a known species of micro-organism in a medium of known composition might prove suitable for the study of the effects of environmental conditions on the organism in such a powder, or such a powder suspended in oil.

Before enquiry into the effects of environmental conditions can be made, certain facts must be established. Organisms suitable for the investigations on hand must be chosen. It must be shown that these organisms can be counted satisfactorily, or at any rate that reasonably concordant replicate viable counts can be obtained and the errors of such counts must be statistically evaluated. It must be shown that the viable organisms counted are uniformly distributed in the powder or that with suitable treatment such uniformity can be attained. The organisms used should remain viable for considerable lengths of time in the powder.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT. PART I

but if they cease to be able to reproduce during the course of the experiment then it must be possible to estimate the extent of such degeneration over the periods of time involved in the experiment. It should clearly be understood that the fact that a particular bacterium does not produce a colony when the medium is plated out may not mean that the organism is dead as an individual, but it does mean that it is incapable of reproduction. In the following work such organisms will, as is usual, be regarded as non-viable.

EXPERIMENTAL TECHNIQUES

Choice of Test Organisms. Considerable thought has been given to the question of the choice of suitable organisms. For the early work on spray-drying it was essential that the test organisms should be non-pathogenic since some of the dried culture might be inhaled. Preliminary experiments with yeasts and moulds did not appear promising, largely on account of difficulties of culture and enumeration. Bacteria, although smaller in size and so more difficult to examine microscopically and distinguish from unorganised matter in total counts, yielded more repeatable figures.

It was considered that both spores and vegetative forms should be examined in this work, for the following reasons. (i) To be satisfactory, suggested processes for sterilisation must destroy the viability of spores, but (ii) a substance which will kill, or even profoundly inhibit vegetative organisms, even if it does not affect spores, may be considered to be satisfactory for the maintenance of sterility in, for example, multiple dose containers. (iii) For the understanding of the life processes of bacteria the behaviour of both spores and vegetative forms is of interest.

Bacillus subtilis appeared to be the most obviously satisfactory non-pathogenic sporing organism. To perform "useful" viable counts on *B. subtilis* in the vegetative form is probably an impossibility owing to the tendency of the cells to form chains and the tendency of these chains to form a matted growth or pellicle on the surface of liquid media. It is difficult, not only to break down the chains into individual cells, but the pellicle is very difficult to wet so that it has proved impossible to obtain the even suspension of organisms necessary for concordant replicate counts. When, however, *B. subtilis* is allowed to spore on the surface of agar each spore is formed individually in a separate bacterial cell. This process is followed by the autolysis of the original vegetative cells; the whole surface becomes moist and the spores can easily be mixed with a suspending fluid. Microscopical examination of the resultant suspension shows the spores to be separate with no tendency to clump. Such a suspension has proved very satisfactory in this work. The details of preparation are as follows.

The organism used was *Bacillus subtilis* (Marburg, No. 3610) obtained from the National Collection of Type Cultures. The surface growth on 10-day agar slopes was washed off with 20 ml. of sterile water, and the resulting suspension was suitably diluted and distributed in glass

ampoules. These were heated at 80°C. for 3 minutes, to destroy any vegetative organisms, and were then stored in a refrigerator. Such suspensions showed no significant decrease in viable count after 6 months' storage.

To find a suitable non-sporing organism has proved to be much more difficult. *Bacterium lactis aerogenes*, which was used in the work described in the previous paper, is non-pathogenic, evidences no marked tendency to chain formation and gives rise to colonies which can easily be counted, but over 99 per cent. of the bacteria are usually destroyed on spray-drying and the survivors fairly rapidly die off in the resultant powder under ordinary conditions of storage. It is possible that some of the difficulties are inherent in the nature of vegetative bacteria. Recently it has been found that *Streptococcus faecalis* appears to be more resistant to drying and storage in the resultant powder, and provided that difficulties involved in obtaining a viable count of a *Streptococcus* can be overcome, this organism may prove very satisfactory. For the present, however, the use of *Bact. lactis aerogenes* has been continued, the strain used being No. 418, obtained from the National Collection of Type Cultures. The details of its use are as follows. For each spray-drying 10 ml. of peptone water was inoculated with the organism and incubated at 30°C. for 24 hours. The suspension so obtained was thoroughly mixed by means of a sterile pipette and added to the solution to be dried.

Choice of Method for Viable Counts. Wilson⁶ advocated a method for counting viable organisms, using roll-tubes which were inoculated by means of dropping pipettes, and the accuracy of the technique has been established by Withell⁷ and others. Anderson and Stuart⁸ and Miles and Misra⁹ used dropping pipettes to perform "surface-viable" counts. By this method surface growths of *B. subtilis* tend to spread, and since only a relatively small number of colonies are counted errors due to interference from spreading are magnified by the large multiplication factor involved. To minimise the difficulty Davis¹⁰, using this method, reduced the colony size by incorporating sodium taurocholate in the medium, but, while he obtained a satisfactory statistical uniformity, he found the salt to have an inhibitory effect. This might operate unequally in the presence of other chemical agents. Such inhibition would be detrimental to the objects of the present investigation. With dropping pipettes as used by the above workers, the size of drop delivered is not only governed by the external diameter of the tip, but is also influenced by the temperature and viscosity of the liquid dropped. They are not, therefore, particularly suitable for measuring samples of liquids where the viscosity may differ, as is the case with original and reconstituted samples before and after drying. Snyder¹¹ compared the use of dropping pipettes with that of graduated pipettes in combination with both roll-tubes and plates and with surface counts. He found graduated pipettes to be more accurate, but the significance of the difference was lost when estimating viable counts because the pipetting

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errors contributed only a small proportion of the total error. It was decided, therefore, to use pipettes marked with two graduations for the delivery of 1 ml. for dilutions and inoculations: roll-tubes were used for the counts, which were carried out in the following manner. 9 ml. of diluent were placed in each of a number of plugged test-tubes. 1 ml. of the suspension to be counted was pipetted into the first of these tubes. A second pipette was used to mix the contents of the tube and to transfer 1 ml. of the mixed suspension to the second tube. A suitable number of such serial dilutions was made, a fresh pipette being used for each transference. 1 ml. portions of the final dilution were inoculated into roll-tubes containing 5 ml. of melted agar medium which had previously been maintained at a temperature of 46°C. The roll-tubes, after inoculation, were held horizontally under a stream of cold water and rotated until the agar set. They were then placed, plug-downward in the incubator. The accuracy of the technique has been assessed statistically according to the method used by Withell⁷, Davis¹⁰, Berry and Michaels¹² and others. In the immediately following paragraphs the results refer to experiments with *B. subtilis* spores. A summary of the corresponding results with *Bact. lactis aerogenes* is given on pages 891 *et seq.*

The Accuracy of the Graduated Pipettes. The pipettes used were made of fine bore glass tubing tapered at one end to a stout point. A ring was marked round the tube about 2 cm. from this end, and a second mark made at a distance above this corresponding to a delivery of 1 ml. The pipettes were calibrated gravimetrically. The weights of water delivered by the pipettes were all greater than 0.9873 and less than 1.0073 g. and the mean deviation from the theoretical values (0.9973 g.) was 0.0043 g., giving a mean percentage deviation of 0.431 per cent. Jennison, Marshall and Wadsworth¹³ stated that pipettes suitable for viable counts should have an accuracy within ± 1 per cent.

In a viable count, however, the delivery of liquid is not controlled as carefully as in the above calibration. Twenty pipettes were taken at random and fitted with rubber teats by means of which water could be drawn up to the upper mark and ejected until the meniscus reached the lower mark. The water so discharged was weighed, and the process repeated 3 times for each pipette. In order to make the conditions as severe as possible no specific time was allowed for drainage, and the various pipettes when being filled were inserted at different depths into the water. The results are given in Table I.

From the weights of water discharged from the same pipette the variance was calculated and from the mean of these the co-efficient of variation was found to be 0.77 per cent. From the same figures three variances of the weights of water discharged from different pipettes were also calculated and from the mean of these the co-efficient of variation was found to be 1.32 per cent. These figures compare with 1.03 per cent. and 1.83 per cent. obtained by Withell⁷, using dropping pipettes.

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The Accuracy of the Dilution Technique. In most "viable counts," 10^{-1} , 10^{-2} , and 10^{-3} , dilutions have been prepared, the last dilution being tubed. Each dilution was prepared by pipetting 1 ml. of suspension into 9 ml. of diluent. As diluting agent quarter-strength Ringer's solution was used with *Bact. lactis aerogenes* while distilled water was used with *B. subtilis* since it was found to have no destructive effect on spores.

TABLE I

ERRORS INVOLVED IN MEASURING 1 ML. OF WATER WITH PIPETTES, FILLING AND EMPTYING BEING EFFECTED BY A RUBBER TEAT

	Weights			Mean (\bar{x})	$S(x-\bar{x})^2$	Variance
	0.9830	0.9825	0.9800	0.9818	0.00000517	0.000002585
	0.9950	0.9920	0.9750	0.9873	0.00023267	0.000116335
	0.9793	0.9687	0.9620	0.9700	0.00015218	0.000076090
	0.9730	0.9809	0.9651	0.9730	0.00012482	0.000062410
	0.9945	1.0055	0.9972	0.9991	0.00006573	0.000032865
	0.9865	0.9700	0.9820	0.9795	0.00014550	0.000072750
	0.9785	0.9560	0.9793	0.9713	0.00034993	0.000174965
	0.9845	0.9822	0.9864	0.9843	0.00000886	0.000004430
	0.9785	0.9934	0.9841	0.9853	0.00011329	0.000056645
	0.9718	0.9867	0.9753	0.9779	0.00012141	0.000060705
	0.9994	0.9831	0.9834	0.9886	0.00017393	0.000086965
	1.0000	1.0100	0.9916	1.0005	0.00016971	0.000084855
	1.0020	1.0032	0.9928	0.9993	0.00006475	0.000032375
	0.9792	0.9960	0.9920	0.9891	0.00015403	0.000077015
	1.0086	0.9925	0.9945	0.9985	0.00015401	0.000077005
	0.9731	0.9807	0.9910	0.9816	0.00016142	0.000080710
	1.0050	1.0058	1.0000	1.0036	0.00001976	0.000009880
	0.9873	0.9841	0.9959	0.9891	0.00007448	0.000037240
	0.9807	0.9800	0.9759	0.9789	0.00001345	0.000006725
	1.0069	1.0131	1.0117	1.0106	0.00002115	0.000010575
Mean (\bar{x})	0.9883	0.9888	0.9858	Total	...	0.001163125
$S(x-\bar{x})^2$	0.00272404	0.00419382	0.00274324	Mean Variance	...	0.000058156
Variance	0.00014337	0.00022073	0.00014438	S. Deviation	...	0.007626
M. Variance	...	0.00016949		Co-efficient of		
S. Deviation	...	0.01302		Variation	...	0.77%
Co-efficient of Variation	...	1.32%				

Wilson¹⁴ showed that distilled water was lethal to vegetative organisms and found quarter-strength Ringer's solution to be satisfactory for suspending such an organism. He also showed that distilled water had a dispersive effect on clumps of bacteria occurring in milk. The 9-ml. quantities of diluent were delivered into the test-tubes from a burette consisting of a graduated 10-ml. pipette, plugged at the upper end with cotton wool, and connected at the lower end by means of a two-way tap, either to a flask containing the diluent, or to a hooded nozzle from which the diluent could be measured into a test-tube. The co-efficient of variation of ten 9-ml. samples delivered from the apparatus was found to be 0.287 per cent.

In order to estimate the overall error of diluting and pipetting, 20 serial dilutions were carried out. From each of 20 spore suspensions, 10^{-1} , 10^{-2} , and 10^{-3} dilutions were prepared in duplicate. Five roll-tubes were then inoculated from each of the 10^{-3} dilutions giving 2 sets of 5 tubes from each spore suspension. The results are given in Table II.

The variance of the mean counts obtained from each pair of dilutions was calculated and from these the mean co-efficient of variation was found to be 3.06 per cent. Another similar experiment by a different

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worker gave a mean co-efficient of 3.37 per cent. These figures may be compared with 4.95 per cent. obtained by Withell⁷ and 3.79 per cent. obtained by Berry and Michaels¹².

The Error of Counting Colonies of B. subtilis. If a roll-tube be counted on successive occasions counts will be obtained which differ slightly from one another. The differences may be attributed to:—(a) Inability to recognise small colonies; (b) The appearance of "double" colonies where two colonies lie one above the other in the medium; (c)

TABLE II
ERRORS OF DILUTING AND PIPETTING

Experiment	1st 10 ⁻³ dilution (mean count of 5 tubes)	2nd 10 ⁻³ dilution (mean count of 5 tubes)	Variance
1	168	163	12.5
2	218	219	0.5
3	199	197	2.0
4	186	195	40.5
5	187	195	32.0
6	206	203	4.5
7	208	201	24.5
8	212	219	24.5
9	196	204	32.0
10	212	226	98.0
11	237	253	128.0
12	131	124	24.5
13	119	128	40.5
14	124	120	8.0
15	159	160	0.5
16	134	131	4.5
17	132	137	12.5
18	135	129	18.0
19	78	72	18.0
20	79	75	8.0

The suppression of some colonies by local crowding; (d) The occurrence of spreading surface colonies both at the medium-air interface and the medium-glass interface; (e) The development of daughter colonies arising from the surface colonies; (f) Parallax error. Colonies which have been counted are identified by marks made on the surface of the glass. As the tubes are rotated, the relative positions of the marks and of the colonies lying deeper in the agar may alter so that colonies which have been marked appear to be unmarked and vice versa.

Wilson¹⁴ investigated the first three of these sources of error. He concluded that the use of a small hand lens magnifying 2 or 3 diameters greatly aided the recognition of small colonies. He also examined the structure of double colonies appearing as circular surface colonies upon which smaller, lenticular colonies, lying deeper in the agar, were superimposed, and suggested that where the lenticular colony was placed centrally with respect to the circular colony these should be regarded as one, whilst when the lenticular colony was eccentrically placed the two colonies should be regarded as separate. Wilson also showed that if the tubes are overcrowded suppression of some colonies may occur and recommended that the count should lie between 30 and 300. All these recommendations have been followed in the work described in this paper.

The presence of spreading surface growth has been generally held to

cause low values for the viable count and various attempts have been made to reduce the tendency of such colonies to form and to reduce their size when they do occur. Thornton¹⁵ endeavoured to control them by reducing the nutrient content of the agar medium but the period of incubation had to be extended to 10 days which is unsuitable for roll-tubes because they dry out unless the incubator atmosphere is kept saturated with water, in which case the surface growth is greatly increased. Various methods of drying the surface of the agar before incubation were tried but all proved unsatisfactory.

The surface growth may affect the count in two ways. Firstly, it may become confluent with other colonies growing on the surface. The proportion of these, however, is small and the surface growth occupies usually only one-third or less of the total surface. The number of colonies affected in relation to the total number occurring in the tube is therefore not likely to be large. Moreover, discrete colonies are often observed lying on the surface in the middle of the spreading growth and surrounded by a clear ring, which suggests that during the period of incubation they have not lost their identity. Secondly a more serious difficulty is caused by the presence at the edge of the spreading growth of numbers of small discrete daughter colonies, produced on the surface from it. These colonies differ in appearance from those submerged colonies normally seen in tubes where no spreading growth occurs; differentiation was based on the following characteristics.

Submerged Colonies are small, irregular, or "woolly" colonies, $\frac{1}{2}$ to 1 mm. in diameter, opaque, white or pale cream by reflected light, brown by transmitted light.

Surface Colonies may be produced from the submerged colonies. When these have grown to reach the upper surface they produce a spreading colony thereon. The size of this depends on the amount of moisture present at the surface of the medium. During incubation this moisture film gradually dries up, so that the depth at which an organism is implanted in the medium and the speed with which it grows govern the size of surface colony produced. Thus many colonies produce no surface growth. Others produce circular surface colonies; these are thin, or slightly raised, the surface being smooth or showing radiate ridges or wrinkles; the submerged colony can be seen as a denser spot lying below the centre of the circular colony. More advanced surface colonies show transitions from the circular type through lobate or pinnatifid forms to much-branched, somewhat radiate, moderately thin, colonies, the ends of the branches and their subdivisions being club-shaped. A small submerged colony can be seen at the centre of radiation. In the largest "spreaders" the centre is occupied by a continuous film of growth. Since these have probably been produced from organisms implanted on or very near the surface of the medium there is no submerged colony apparent. Surface colonies are occasionally produced at the medium-glass interface. These are very thin with irregular edges and are of an even, granular texture.

Daughter Colonies are sometimes produced from the edges of extensive .

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surface "spreaders." They are circular or scaly, effuse and much thinner than the "spreader" or the circular colonies described above. Since they are not produced from submerged colonies they show no central spot. They occur in groups the members of which are similar in size and texture and the presence of submerged colonies growing below them can usually be easily recognised. Daughter colonies may also be formed from colonies at the medium-glass interface. They resemble their parent colonies but occur in well-marked groups.

It appeared that it should be possible to perform a "viable count" satisfactorily even in the presence of the surface growths. Counting tests were therefore performed to determine the effect of these growths and of the parallax error.

Twenty tubes were taken and each was counted three times. Three methods of performing the count were used, as follows:—

Method (1). 8 longitudinal lines and 3 transverse rings were marked on each tube with a wax pencil. The tube was thus divided into small areas within which the colonies were counted. Colonies touching the line were counted only on the upper and right hand margins of each area. All colonies including indistinct and daughter colonies were counted.

Method (2). As above, but any daughter colonies or colonies rendered indistinct by the surface growth were ignored. A new set of tubes was used, half the tubes having a high count and half a lower count.

Method (3). As in (2) but marking the tubes with 1 longitudinal line and 6 transverse rings.

The results are set out in Table III.

TABLE III
THE ERRORS OF COUNTING TUBES ON SUCCESSIVE OCCASIONS BY DIFFERENT METHODS

Method (1)		Method (2)		Method (3)	
Counts	Variance	Counts	Variance	Counts	Variance
347 350 346	4.5	436 431 432	7.0	433 426 430	12.5
610 599 617	81.5	463 465 475	41.5	463 453 465	41.5
325 324 322	3.5	404 389 419	226.5	409 407 409	1.5
563 566 561	6.5	448 422 437	170.5	393 394 393	7.0
546 549 527	142.5	403 384 394	145.0	443 459 443	85.5
598 590 596	17.5	464 448 453	65.0	400 400 408	21.5
537 522 486	687.0	430 385 399	310.5	408 395 394	61.0
400 402 398	4.0	431 395 402	364.5	394 399 401	13.0
457 434 432	176.5	194 189 191	281.5	431 429 431	2.5
369 364 374	25.0	167 170 165	6.5	193 194 193	0.5
344 356 356	48.0	421 393 391	6.5	172 173 171	1.0
339 334 331	18.0	196 192 188	7.0	189 199 192	26.5
339 332 334	76.5	202 198 191	9.5	197 187 182	58.5
273 275 290	253.0	193 196 188	16.5	215 225 223	91.0
305 277 278	86.5	177 177 181	5.5	194 188 188	28.0
277 265 273	37.5	186 184 189	16.5	178 178 178	0.0
249 282 287	426.5	206 209 207	2.5	193 184 186	22.5
281 271 276	25.0	211 210 209	1.0	196 201 195	11.5
272 257 272	75.0			205 214 210	20.5
275 273 277	4.0				
	2198.5		1708.0		518.0
Overall variance	= 109.9	Overall variance	= 85.4	Overall variance	= 25.9
Standard deviation	= 10.48	Standard deviation	= 9.24	Standard deviation	= 5.09
S.E. of mean of three counts	= 6.098	S.E. of mean of three counts	= 5.34	S.E. of mean of three counts	= 2.94

The Standard Error of the Mean of Three Counts using Method (1) is 6.098, and the majority of the variances are excessive. The effect of the surface growth, either by virtue of obliteration of other colonies or by masking of them at its fringes, would be greatest on the lower counts. The use of Method (2) should minimise the effect. On the other hand, the parallax error should not be considerable on tubes of low count, for the distance separating the colonies is greater and the number of colonies bordering the demarkation lines fewer than in tubes of high count. The experimental results of Method (2) are in agreement with this, the variances of the lower counts being significantly improved, while those of the higher counts show no such improvement. Using Method (3) the parallax error has been minimised, for this occurs almost entirely along the longitudinal markings, since a slight rotation of the tube easily brings a colony from one side of the line to the other. Using this method the variances of both high and low counts were satisfactory and the Standard Error of the Mean of Three Counts was found to be 2.94, comparing favourably with that of 2.44 obtained by Berry and Michaels¹² using *E. coli*. It may be mentioned that a different worker using Method (2) obtained a Standard Error of 4.73. The same worker using Petri dishes instead of roll-tubes obtained the figure of 7.26 for the Standard Error.

A direct estimate of the effect of the surface growth on the viable count was also afforded by a series of tubes inoculated from the same suspension, in which a considerable variation occurred in the size of surface colonies present. The results are given in Table IV.

TABLE IV
THE EFFECT OF SURFACE GROWTH ON THE VIABLE COUNT OF *B. SUBTILIS*

Count	Area of Surface Growth	Count	Area of Surface Growth
146	Extensive	143	Very slightly
137	Nil	111	Slight
133	Moderate	143	Nil
123	Slight	146	Nil

The highest count was obtained in two tubes, one of which bore an extensive surface growth, while the other showed none at all, and the tube with the lowest count had only a small "spreader." There is, in fact, no significant difference between the counts which can be attributed to the effect of the surface growth, and it appears that viable counts may be satisfactorily performed even in the presence of extensive spreading colonies.

The Suitability of the Agar Medium. As pointed out above, an organism is regarded as viable if it produces a colony in the roll-tube, and as non-viable if it fails to produce such a colony. The composition of the medium used in the roll-tubes is to some extent responsible for the rate of growth of the colonies and the ultimate size which they attain. It is even more important that certain, possibly damaged or weakened, individual cells may fail to grow in one medium, while they produce a

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colony and so give rise to a higher count in a more favourable substrate. The medium is, therefore, very important. For work such as that described in this paper it should (i) be accurately reproducible, (ii) be uniform throughout, all the ingredients preferably having been in solution, (iii) give concordant replicate counts from time to time and from batch to batch, (iv) give maximal counts for any given bacterial suspension.

Broadly speaking, three types of media are available: (i) Synthetic media prepared entirely from pure chemicals. Different batches may be identical, but they are difficult to prepare and opinion is by no means fixed as to the proportions of ingredients which they ought to contain. Their sensitivity is likely to vary greatly with the addition or omission of trace substances or vitamins. (ii) At the other extreme are the media prepared by direct extraction of meat tissues, with or without addition of serum. Such media are very sensitive, often giving the highest counts, but there is no guarantee of their constancy of composition from batch to batch. (iii) In between the above classes are the media prepared from peptone, with or without addition of commercial meat extracts. These have the advantages of being moderately sensitive and yet reproducible, for relatively large samples of peptone and meat extract can be purchased so that the batches of media prepared from them during the course of a lengthy set of experiments will not vary detectably. For these reasons this type of medium was chosen and three examples were examined. They consisted of:—

- A. 3 per cent. of agar with 2.0 per cent. of peptone and 0.5 per cent. of sodium chloride.
- B. The same with the addition of 0.5 per cent. of proteolysed liver extract.
- C. The same as A with the addition of 0.5 per cent. of Lab-Lemco.

To prepare the media the ingredients were dissolved in distilled water, adjusted to pH 7.6 with N caustic soda, solution being effected by heating in an autoclave at 10 lb. pressure. The solution was filtered through washed sand and filter-paper pulp. The medium was then sterilised by autoclaving at 10 lb. pressure. The final pH was 7.2.

To compare the media, 25 roll-tubes of each were inoculated with 1 ml. from the same suspension of the test organism. Each of these batches was divided into 5 groups of 5 tubes which were incubated at different temperatures, the same 5 temperatures being applied to all three batches. The results are given in Table V.

The mean counts obtained with medium C are lower than those obtained with the other media. The counts on Medium A at temperatures between 26°C. and 42°C. do not differ significantly from those on Medium B, but the surface growth was thicker on the latter. Medium A was, therefore, chosen for use with *B. subtilis*.

Thornton¹⁵ suggested that batches of media should comply with two requirements: (i) Different batches should give reproducible results. (ii) Parallel platings from the same batch should develop the same number of colonies within the limits of sampling variance.

TABLE V
THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT OF *B. SUBTILIS*

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	90	131	155	127	120	79	112	136	198	75	53	72	77	82	121
	109	136	135	133	122	36	110	126	139	94	56	95	88	89	126
	86	118	120	120	127	7	131	116	107	115	57	73	77	83	138
	103	111	122	142	138	10	149	125	112	85	57	95	127	97	103
	86	141	145	106	108	22	124	137	124	97	44	80	114	93	117
Mean ...	95	127	135	126	123	17	125	128	116	93	53	83	97	89	121

To test requirement (i), 5 tubes were rolled from each, using the same suspension of the test organism in each case. The results for successive batches are given in Table VI.

TABLE VI
THE REPRODUCIBILITY OF VIABLE COUNTS ON SUCCESSIVE BATCHES OF MEDIA

Batch I					Batch II						
Count					Count					T	P
82	90	76	86	84	118	71	92	85	91	0.462	0.6 to 0.7
97	146	112	111	116	115	123	123	119	119	0.363	0.7 to 0.8
126	140	127	130	110	125	113	120	133	110	0.942	0.3 to 0.4

The probability is in each case satisfactory and it may be concluded that different batches of the medium can be prepared having the same sensitivity to the test organism.

Requirement (ii) can be tested by the use of the statistic χ^2 calculated in the form

$$\chi^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$$

Berry and Michaels¹² tested each batch of medium by counting 20 replicate tubes and comparing the value of χ^2 obtained from them with that to be expected if the variation involved only the normal sampling variance. For comparison, results of similar tests are included here. Table VII shows a typical result for one batch and Table VIII shows the summarised results for all the batches used in the present experiments. In all cases P was found to be satisfactory.

This test, however, is really a test of all the errors involved in carrying out the count and while, assuming all other errors to be small, the agar may be assumed to be satisfactory, the test is not sufficiently comprehensive to assess the overall error. Moreover, such a test would conceiv-

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be of use in testing media used with mixed bacterial cultures where, for instance, the encouragement of certain fast-growing organisms might militate against the development of slower growing colonies, but it is difficult to see how a homogeneous medium could increase the variance of counts on a pure culture.

The Duration and Temperature of Incubation. The optimum temperature for growth of *B. subtilis* is given by Bergey¹⁶ as 30° to 37°C. and by Topley and Wilson¹⁷ as 37°C. It is possible also that it may vary to some extent with the medium employed and a test was therefore carried out to examine this. Table V, to which reference has already been made, shows the counts resulting from incubation at various temperatures and on various media. In these experiments the temperature for maximal counts for *B. subtilis* appears to lie between 26°C. and 42°C. and incubation temperatures within this range were therefore used for this organism.

TABLE VII
GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF
B. SUBTILIS

Count (x)	Mean (\bar{x})	$(x - \bar{x})$	$(x - \bar{x})^2$	$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}}$
180	193	13	169	2782 <hr/> 193 = 14.42
181		12	144	
206		13	169	
192		1	1	
195		2	4	
180		13	169	
206		13	169	
181		12	144	
189		4	16	
207		14	196	
203		10	100	
183		10	100	
180		13	169	
202		9	81	
211		18	324	
203		10	100	
190		3	9	
180		13	169	
178		15	225	
211	18	324		
N = 19 $\chi^2 = 14.42$ P = 0.8 — 0.7				

TABLE VIII
SUMMARY OF VALUES OF χ^2 OBTAINED FROM SETS OF 20 ROLL-TUBES OF
B. SUBTILIS

Batch No	No. of Tubes	N	χ^2	P
1	20	19	14.42	0.8—0.7
2	20	19	17.98	0.7—0.5
3	20	19	14.13	0.8—0.7
4	20	19	13.17	0.9—0.8
5	20	19	15.60	0.7—0.5
6	20	19	18.59	0.5—0.3
7	20	19	11.83	0.9—0.8
8	20	19	14.81	0.8—0.7
9	19	18	11.83	0.9—0.8

Thornton¹⁵ suggested that batches of media should comply with two requirements: (i) Different batches should give reproducible results. (ii) Parallel platings from the same batch should develop the same number of colonies within the limits of sampling variance.

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	90	131	155	127	120	79	112	136	198	75	53	72	77	82	121
	109	136	135	133	122	36	110	126	139	94	56	95	88	89	126
	86	118	120	120	127	7	131	116	107	115	57	73	77	83	138
	103	111	122	142	138	10	149	125	112	85	57	95	127	97	103
	86	141	145	106	108	22	124	137	124	97	44	80	114	93	117
Mean ...	95	127	135	126	123	17	125	128	116	93	53	83	97	89	121

To test requirement (i), 5 tubes were rolled from each, using the same suspension of the test organism in each case. The results for successive batches are given in Table VI.

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126	140	127	130	110	125	113	120	133	120	0.942	0.3 to 0.4

The probability is in each case satisfactory and it may be concluded that different batches of the medium can be prepared having the same sensitivity to the test organism.

Requirement (ii) can be tested by the use of the statistic χ^2 calculated in the form

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The Duration and Temperature of Incubation. The optimum temperature for growth of *B. subtilis* is given by Bergey¹⁶ as 30° to 37°C. and by Topley and Wilson¹⁷ as 37°C. It is possible also that it may vary to some extent with the medium employed and a test was therefore carried out to examine this. Table V, to which reference has already been made, shows the counts resulting from incubation at various temperatures and on various media. In these experiments the temperature for maximal counts for *B. subtilis* appears to lie between 26°C. and 42°C. and incubation temperatures within this range were therefore used for this organism.

TABLE VII
GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF
B. SUBTILIS

Count (x)	Mean (\bar{x})	(x - \bar{x})	(x - \bar{x}) ²	$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}}$
180	193	13	169	2782 193 =14.42
181		12	144	
206		13	169	
192		1	1	
195		2	4	
180		13	169	
206		13	169	
181		12	144	
189		4	16	
207		14	196	
203		10	100	
183		10	100	
180		13	169	
202		9	81	
211		18	324	
203		10	100	
190		3	9	
180		13	169	
178		15	225	
211		18	324	

$$N = 19 \quad \chi^2 = 14.42 \quad P = 0.8 - 0.7$$

TABLE VIII
SUMMARY OF VALUES OF χ^2 OBTAINED FROM SETS OF 20 ROLL-TUBES OF
B. SUBTILIS

Batch No.	No. of Tubes	N	χ^2	P
1	20	19	14.42	0.8-0.7
2	20	19	17.98	0.7-0.5
3	20	19	14.13	0.8-0.7
4	20	19	13.17	0.9-0.8
5	20	19	15.60	0.7-0.5
6	20	19	18.59	0.5-0.3
7	20	19	11.83	0.9-0.8
8	20	19	14.81	0.8-0.7
9	19	18	11.83	0.9-0.8

In order to determine the incubation period for *B. subtilis* likely to produce the most reliable counts 5 tubes were incubated at 32°C. and counted at suitable intervals. The results are given in Table IX.

TABLE IX
EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *B. SUBTILIS*

Period of Incubation	Count	Mean	Variance
24 hours ...	160, 165, 143, 161, 115, 148	149	343
40 hours ...	168, 163, 145, 160, 150, 146	155	93
48 hours ...	171, 166, 146, 154, 150, 151	156	98
60 hours ...	175, 155, 146, 152, 148, 143	153	133

The mean counts show no significant differences but at 24 hours the variance of the counts appears excessive, probably because many of the colonies are too small to be seen satisfactorily. While the experiment is too small to permit of accurate conclusions, the mean count at 24 hours would appear to be unreliable. After 60 hours' incubation the surface growth is thicker and, while this has produced no significant diminution in count, it renders the process of counting more difficult. The most suitable incubation period for *B. subtilis* would therefore appear to be about 48 hours. The fact that the count has not diminished after 60 hours tends to confirm the findings discussed above that the surface growth has little effect upon the viable count.

Table X shows the result of another experiment in which temperature and duration of incubation were both varied. It suggests that a temperature between 32°C. and 40°C. should be used with an incubation period of about 48 hours.

TABLE X
EFFECT OF TEMPERATURE AND DURATION OF INCUBATION ON THE VIABLE COUNT OF *B. SUBTILIS*

Period of Incubation	Temperature							
	26° C.		32° C.		37° C.		40° C.	
	Count	Mean	Count	Mean	Count	Mean	Count	Mean
24 hours ...	65, 58, 50, 55, 45	65	188, 192, 206, 220, 185	196	336, 246, 307, 245, 410	309	367, 414, 314, 429, 416	382
48 hours ...	89, 119, 105, 108, 111	106	222, 205, 223, 221, 242	223	442, 301, 369, 356, 420	374	401, 470, 345, 446, 405	393
120 hours ...	107, 154, 142, 130, 140	135	235, 226, 242, 217, 210	226	385, 330, 333, 346, 410	361	411, 378, 383, 392, 405	394
168 hours ...	105, 151, 141, 118, 131	129	240, 227, 235, 226, 221	230	390, 315, 326, 350, 411	357	397, 457, 383, 385, 407	386

The Normal Sampling Variance. Fisher, Thornton and Mackenzie¹⁸ showed that plate counts approximated to small samples of a Poisson series and deduced from this that x^2 , the Index of Dispersion, should exhibit a characteristic distribution which was tabulated by Elderton¹⁹. The distribution of the values of x^2 determined experimentally could, then be compared with the hypothetical distribution, and the Goodness

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of Fit determined. Such a comparison can be used to examine the overall errors of performing viable counts and provides a more critical test than the use of a single χ^2 . The values of χ^2 obtained with 100 samples of spore suspensions of *Bacillus subtilis* have been tabulated in Table X and their Goodness of Fit tested.

The observed distribution of χ^2 shows no significant departure from the theoretical distribution and the Probability obtained is satisfactory. It may be concluded, therefore, that the technique used is reliable and that accurate and reproducible results can be obtained with it.

Results of Test of Counting Technique using Bact. lactis aerogenes. Tables XI, XII, XIII, XIV and XV relating to *Bact. lactis aerogenes* correspond with Tables III, V, VII, IX and X respectively for *B. subtilis*. Table XVI shows that quarter-strength Ringer's solution is a suitable diluent for use with *Bact. lactis aerogenes*. These tables establish the fact that reliable and reproducible values for the viable count of this organism can be obtained using Medium A and incubating at 20°C. to 40°C. for 24 hours.

TABLE X
GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS ON SETS OF
FIVE ROLL-TUBES, USING *B. SUBTILIS*

Value of χ^2	Expected Frequency (m)	Observed Frequency (m+x)	Difference (x)	$\frac{\chi^2}{m}$
Under 1	9.02	13	3.98	1.756
Between 1 and 2	17.40	11	-6.40	2.355
Between 2 and 3	17.79	19	1.21	0.082
Between 3 and 4	15.18	20	4.82	1.530
Between 4 and 5	11.87	9	-2.87	0.695
Between 5 and 6	8.82	8	-0.82	0.076
Between 6 and 7	6.33	6	-0.33	0.017
Between 7 and 9	7.48	8	0.52	0.036
Over 9	6.11	6	-0.11	0.002
$\chi^2 = 6.549 \quad N = 8 \quad P = 0.5-0.7$				

This result was confirmed by another worker, who obtained the following results: $-\chi^2=6.571, N=7, P=0.3-0.5$.

TABLE XI
THE ERRORS OF COUNTING TUBES ON SUCCESSIVE OCCASIONS BY METHOD (III)

Counts	Variance	Counts	Variance
183 190 187	12.5	238 236 240	4.0
203 206 211	6.5	218 214 219	7.0
201 205 193	37.5	216 211 215	7.0
209 207 207	1.5	237 234 244	26.5
210 206 208	4.0	224 224 218	12.0
182 175 180	13.0	230 233 228	7.0
209 209 210	0.5	200 202 201	1.0
206 205 202	4.5	188 189 183	0.5
203 203 203	0.0	205 200 207	13.0
221 218 218	3.0		
232 231 229	2.5		

Overall Variance = 8.175
Standard Deviation = 2.86
S.E. of Mean of Three Counts = 1.65

TABLE XII

THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	595	575	659	609	591	544	553	620	601	614	416	531	403	499	306
	644	610	679	608	603	596	566	706	616	596	325	507	403	504	319
	621	550	660	567	603	555	574	616	657	650	308	498	452	429	391
	636	583	658	657	681	591	586	572	564	653	321	413	414	501	398
	658	541	693	574	591	644	564	659	663	—	365	549	433	481	396
Mean ...	631	572	670	603	614	586	569	635	620	628	347	499	421	483	362

At 18 hours many colonies are too small easily to be seen. At 48 hours large colonies show "tailing." At 24 hours colonies are all discrete and can be distinguished with ease.

TABLE XIII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF *BACT. LACTIS AEROGENES*

Count (x)	Mean (\bar{x})	(x - \bar{x})	(x - \bar{x}) ²	$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}}$
220	211	9	81	$\frac{4705}{211} = 22.3$
230		19	361	
224		13	169	
197		14	196	
203		8	64	
208		3	9	
191		20	400	
177		34	1156	
244		33	1089	
209		2	4	
201		10	200	
228		17	289	
205		6	36	
235		24	576	
207		4	16	
207		4	16	
209		2	4	
202		9	81	
218		7	49	
208		3	9	
$\chi^2 = 22.3 \quad N = 19 \quad P = 0.2 - 0.3$				

TABLE XIV

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *BACT. LACTIS AEROGENES*

Period of Incubation	Count						Mean	Variance
18 hours ...	34	43	37	40	36		38	50
24 hours ...	32	41	37	40	36		37	51
48 hours ...	32	42	38	38	36		37	53

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TABLE XV

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS ON SETS OF FIVE ROLL-TUBES USING *BACT. LACTIS AEROGENES*

Value of χ^2	Expected frequency (m)	Observed frequency (m + χ)	Difference (χ)	$\frac{\chi^2}{m}$
Under 1	8.48	11	2.52	0.75
Between 1 and 2	16.36	20	3.64	0.81
" 2 and 3	16.72	17	0.28	0.01
" 3 and 4	14.26	13	-1.26	0.11
" 4 and 5	11.15	10	-1.15	0.12
" 5 and 6	8.26	4	-4.26	2.20
" 6 and 7	5.94	5	-0.94	0.15
" 7 and 9	7.03	9	1.97	0.55
Over 9	5.74	5	-0.74	0.10

$$\chi^2 = 4.80.$$

$$N = 8.$$

$$P = 0.7-0.8.$$

TABLE XVI

THE NON-DESTRUCTIVE ACTION OF QUARTER-STRENGTH RINGER'S SOLUTION ON *BACT. LACTIS AEROGENES*

Period of Exposure (Minutes)					
	0	30	60	120	180
Replicate Counts	436	398	405	398	398
	403	331	365	402	418
	422	359	383	363	357
	407	406	402	431	364
	400	390	388	367	408
Mean,	416	377	389	392	389

RESULTS WITH DRIED POWDERS

The Preparation of the Spray-Dried Powders. The technique of spray-drying and its use for the preparation of powders containing viable organisms have been described by Bullock and Lightbown⁵. In the present work the substrate used was 4 per cent. peptone water. This was adjusted to pH 7.6, filtered distributed in bottles of 1-l. capacity and sterilised by autoclaving. The contents of the bottles were inoculated with a quantity of the suspension of the test organism (spore suspension in the case of *B. subtilis*) calculated to give rise to a count of approximately 2×10^5 per ml. of peptone water. The resultant suspension, cooled in ice, was then spray-dried using an air inlet temperature of 180° to 190°C. for *B. subtilis* and of 70° to 80°C. for *Bact. lactis aerogenes*. Free-flowing powders were obtained in each case. These were stored over phosphorus pentoxide in a desiccator.

Reconstitution of the Bacterial Suspension. A weighed quantity of the powder obtained was dissolved in about 9 ml. of diluent (glass-distilled water in the case of *B. subtilis* and quarter strength Ringer's solution for *Bact. lactis aerogenes*). Bullock and Lightbown⁵ compared the strength of this reconstituted solution with that of the original by estimating the chloride content of each. In the present case, however, the substrate was

TABLE XII

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	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	595	575	659	609	591	544	553	620	601	614	416	531	403	499	306
	644	610	679	608	603	596	566	706	616	596	325	507	403	504	319
	621	550	660	567	603	555	574	616	657	650	308	498	452	429	391
	636	583	658	657	581	591	586	572	564	653	321	413	414	501	398
	658	541	693	574	591	644	564	659	663	—	365	549	433	481	396
Mean ...	631	572	670	603	614	586	569	635	620	628	347	499	421	483	362

At 18 hours many colonies are too small easily to be seen. At 48 hours large colonies show "tailing." At 24 hours colonies are all discrete and can be distinguished with ease.

TABLE XIII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF *BACT. LACTIS AEROGES*

Count (x)	Mean (\bar{x})	$(x - \bar{x})$	$(x - \bar{x})^2$	$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}}$
220	211	9	81	$\frac{4705}{211} = 22.3$
230		19	361	
224		13	169	
197		14	196	
203		8	64	
208		3	9	
191		20	400	
177		34	1156	
244		33	1089	
209		2	4	
201		10	200	
228		17	289	
205		6	36	
235		24	576	
207		4	16	
207		4	16	
209		2	4	
202		9	81	
218		7	49	
208		3	9	
$\chi^2 = 22.3 \quad N = 19 \quad P = 0.2-0.3$				

TABLE XIV

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *BACT. LACTIS AEROGES*

Period of Incubation					Count	Mean	Variance
18 hours	34 43 37 40 36	38	50
24 hours	32 41 37 40 36	37	51
48 hours	32 42 38 38 36	37	53

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TABLE XIX

MORTALITY OF *B. LACTIS AEROGENES* (SUSPENDED IN 4 PER CENT. PEPTONE WATER) DURING SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
70°C	98-40	120°C.	99-95
75°C	99-38	150°C.	99-68
75°C	99-37	180°C.	99-61
80°C	99-90		

The Distribution of Organisms in the Powder.—To test the distribution of the organisms in the powders obtained by spray-drying, 10 samples of powder were weighed out and dissolved in quantities of diluent proportional to their weights, so that the resultant solutions contained equal concentrations of peptone. The weights of powder taken were such that the solutions obtained from them gave a count of about 200,000 per ml. The 10⁻³ dilution from each sample was then plated out, in quintuplicate and the counts determined, as described in the earlier part of this paper. These were compared by means of the Analysis of Variance. The counts obtained with *B. subtilis* are recorded in Table XX and the Analysis of Variance of these in Table XXI.

The results for a similar experiment using *Bact. lactis aerogenes* are given in Table XXII and the corresponding Analysis of Variance in Table XXIII.

TABLE XX
QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*B. SUBTILIS*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.)	5198	5615	5311	4954	5131	6555	5295	4860	6582	5095
Volume (ml.)	8.8	9.5	9.0	8.4	8.7	11.1	8.95	8.2	11.2	8.65
Counts	249 230 260 234 238	224 238 227 227 262	232 236 211 223 233	210 247 244 245 207	228 241 233 220 278	233 256 205 246 216	252 264 227 248 239	250 253 232 233 233	247 226 230 223 236	247 231 241 224 214
Total Counts	1211	1178	1135	1153	1200	1156	1230	1203	1162	1157

TABLE XXI
ANALYSIS OF VARIANCE OF QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*B. SUBTILIS*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	1710.9	9	190.1		
Difference between individuals (error)	9859.6	40	246.5	1.296	
Total	11570.5	49			> 0.2

required to be of minimal chloride content for use in subsequent experiments. The two solutions were, therefore, compared by a colorimetric method. The powder was dissolved in such a quantity of diluent as would produce a solution stronger than the original solution from which the powder was prepared. 4 ml. of this reconstituted suspension was transferred by means of a sterile pipette to one cup of a Spekker photo-electric absorptiometer; 6 ml. of the original suspension was placed in the other cup of the absorptiometer. The two solutions were compared using a dark-blue filter and water was measured into the reconstituted suspension until it matched the original. Mixing of the diluted suspension was effected by means of a platinum wire and the extent of the dilution was noted. The remainder of the reconstituted suspension was diluted proportionately with the sterile diluent. During the process of spray-drying some darkening of the peptone may occur. This would affect the colorimetric reconstitution, increasing the dilution of the reconstituted suspension and causing it to have a lower count. This effect was investigated by spray-drying a 4 per cent. solution of peptone containing 0.5 per cent. of sodium chloride. The relative strengths of the original solution and a reconstituted sample were then determined both by the colorimetric method and by estimation of the chloride content. The results are given in Table XVII.

It was considered established that the colorimetric method was sufficiently accurate.

Percentage mortality of the organism on drying. To determine the effects of spray-drying on the organism in suspension viable counts were carried out on the suspensions fed to the dryer and the material reconstituted as described in the previous paragraph. As will be seen from Tables XVIII and XIX, *B. subtilis* spores suffer up to 10 or 12 per cent. mortality, while only 0.05 to 1.6 per cent. of the *Bact. lactis aerogenes* survive.

TABLE XVII

	Weight Taken (g.)	Volume of Water (ml.)	Colorimetric Factor	Back Titre of Ammonium Thiocyanate Solution	Titre of Sample	Chemical Factor	Error of Colorimetric Method
Original ...	—	—	1.000	5.5 ml.	15.55 ml.	1.000	—
Sample 1 ...	0.7777	15.0 ml.	1.000	3.2 ml.	14.65 ml.	0.945	-5.5 per cent.
Sample 2 ...	0.7563	14.0 ml.	1.175	1.8 ml.	17.45 ml.	1.123	-5.4 per cent.

20 ml. of Silver Nitrate Solution \equiv 21.05 ml. of Ammonium Thiocyanate Solution

TABLE XVIII

PERCENTAGE OF *B. SUBTILIS* SPORES (SUSPENDED IN 4 PER CENT. PEPTONE WATER) KILLED BY SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
134°C.	12.2	165°C.	0.0
150°C.	0.76	181°C.	3.6
150°C.	11.7	205°C.	0.0

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TABLE XXV

ANALYSIS OF VARIANCE OF QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	3825.5	9	425.05	4.71	<0.01
Difference between samples	3604.8	40	90.12		
Total	7430.3	49			

TABLE XXVI

QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED AND MIXED POWDER (*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.)2385	.2640	.2570	.2605	.3000	.2895	.2870	.3850	.4230	.3250
Volume (ml.)	7.0	7.75	7.55	7.65	8.8	8.5	8.4	11.3	12.4	9.55
Counts	76 80 95 80 85	91 86 89 71 94	91 106 93 110 112	109 89 119 76 94	56 92 95 88 98	82 93 100 91 72	81 103 106 112 97	112 114 72 97 86	91 102 104 89 105	88 88 95 118 71
Total Counts	416	431	512	487	429	438	499	481	491	460

TABLE XXVII

ANALYSIS OF VARIANCE OF QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	2097.9	9	233.10	1.184	>0.2
Difference between individuals (error)...	7877.4	40	196.93		
Total	9975.3	49			

Tables XXVI and XXVII show that after thorough mixing by the above method the organisms have become evenly distributed in the powder.

The Viability of Organisms in Stored Powders.—The powders obtained by spray-drying were stored over phosphorus pentoxide in desiccators at room temperature. Samples were weighed out at intervals and dissolved in quantities of diluent proportional to their weight. The resultant suspensions were suitably diluted and the final dilutions were plated out in quintuplicate (10^{-3} for *B. subtilis*, 10^{-2} for *Bact. lactis aerogenes*). The mean count was determined for each set of 5 tubes and these means are set out in Table XXVIII.

Table XXIII suggests that the organisms are not very evenly distributed in the powder. A further spray-drying was performed and samples taken and counted as before. The results are given in Table XXIV and the Analysis of Variance in Table XXV.

TABLE XXII
QUINTUPPLICATE PLATING OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER
(*BACT. LACTIS AEROGENES*)

Sample ...	I	II	III	IV	V	VI	VII	VIII
Weight (g.) ...	·5284	·4680	·5044	·4991	·5061	·4331	·4037	·5702
Volume (ml.) ...	13·21	11·7	12·61	12·48	12·4	10·83	10·09	13·01
Counts	160	191	151	183	142	171	198	178
	181	163	149	187	154	166	138	149
	163	173	152	177	146	141	167	149
	180	158	140	152	148	150	189	134
	180	163	154	188	158	163	184	159
Total Counts	864	848	746	887	748	791	876	759

TABLE XXIII
ANALYSIS OF VARIANCE OF QUINTUPPLICATE PLATING OF 8 SAMPLES OF THE
SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples ...	4100	7	585·7	2·505	0·01—0·05
Difference between individuals . .	7482	32	233·8		
Total ...	11582	49			

Table XXV shows that the organisms in the powder obtained from the drying of a suspension of *Bact. lactis aerogenes* were distributed very unevenly. The remainder of the powder was therefore placed in a sterile vaccine bottle together with some sterile glass beads. The bottle was closed by means of a rubber cap and fixed to a revolving wheel by means of which it was slowly rotated for a period of 24 hours. 10 further samples were then taken and viable counts performed as before. These are given in Table XXVI and the Analysis of Variance in Table XXVII.

TABLE XXIV
QUINTUPPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER
(*BACT. LACTIS AEROGENES*)

sample ...	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·3170	·2600	·2345	·2555	·2075	·2160	2355	·1830	·2215	·3305
Volume (ml.) . .	9·0	7·4	6·65	7·25	5·9	6·15	6·7	5·2	6·3	9·4
Counts	53	54	55	40	46	58	53	35	42	35
	59	86	47	38	51	53	45	39	41	45
	36	71	54	48	43	49	55	34	49	46
	52	77	53	49	56	44	49	45	44	46
	53	71	60	31	53	74	61	51	64	44
Total Counts ...	253	359	269	206	249	278	263	204	240	207

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in further experiments. Such experiments involving the exposure to antiseptics and heat of both the powder itself and the powder suspended in oil, are in progress in this department.

In the case of *Bact. lactis aerogenes* the circumstances are not so simple. Table XIX shows that between 98.4 and 99.9 per cent. of the organisms are killed, i.e., that 0.1 to 1.6 per cent. survive, a very great variation. Furthermore, Table XXVIII shows that the organisms die off rapidly in the dry powder, a result previously reported by Bullock and Lightbown⁵, who also showed that *Bact. lactis aerogenes* even in powder form is considerably more sensitive to heat than the spores of *B. subtilis*. Thus we should expect that slight variations in drying conditions around the spray jet and in the time during which the various portions of powder are in contact with the metallic surfaces of the drying chambers would have a considerable effect on the number of organisms surviving. It is not therefore surprising to find that as shown in Tables XXIII and XXV there is evidence of uneven distribution of the organisms in the dried powder as discharged from the dryer. However, the powder is light and easily mixed and this treatment is shown in Table XXVII to result in a powder in which the organisms are evenly distributed.

It would appear, therefore, that the satisfactory nature or otherwise of a spray-dried powder probably depends upon the resistance of the organism to the drying process. If the organism is resistant, as in the case of *B. subtilis* spores, the powder is eminently satisfactory for further work. If the organism is susceptible as in the case of *Bact. lactis aerogenes*, then the powder is not so suitable. It requires careful mixing and in examining the effects of heat or antiseptics on the powder, and powder suspended in oil, the high death rate normally associated with the organisms must be taken into account. Experiments are therefore in progress to see if a more suitable organism than *Bact. lactis aerogenes* can be found and some, more promising, results have been obtained with *Streptococcus faecalis*. Meanwhile it can be said that a powder containing *Bact. lactis aerogenes* in even distribution can be obtained and is suitable for use in experiments concerned with the study of environmental conditions on a relatively sensitive organism.

SUMMARY

1. The technique of performing viable counts using graduated pipettes with roll-tubes has been examined and the accuracy of the method has been assessed by statistical analysis.
2. It has been shown that satisfactory viable counts can be obtained of spores of *B. subtilis* in suspension or in powders. The spreading surface growth of the organism has been shown to have no significant effect upon the count and roll-tube counts may be performed satisfactorily in its presence.
3. It has been shown that if even suspensions of *B. subtilis* spores are spray-dried the viable organisms are evenly distributed in the resultant powder.

TABLE XXVIII

EFFECT OF STORAGE ON COUNT OF ORGANISMS CONTAINED IN SPRAY-DRIED POWDERS

<i>B. subtilis</i>								
Period of Storage (Days)	0	12	22	48	80	124	157	
Mean Count of 5 Tubes	129	124	126	132	131	132	128	

<i>Bact. lactis aerogenes</i>								
Period of Storage (Days)	0	6	7	9	13	21	37	
Mean Count of 5 Tubes	5426	743	513	309	301	200	64	

DISCUSSION

Viable counts have always been subject to much criticism. In the last few decades some of the objections have been overcome. As a result of carrying out a large number of counts and submitting the results to statistical analysis it has been shown that, with certain organisms and using particular techniques, counts may be performed so as to give results reproducible within certain ascertained limits of error. Methods have also been elaborated for establishing the suitability or otherwise of particular media. In the present work *B. subtilis* spores and *Bact. lactis aerogenes* non-sporing organisms have been submitted to this type of examination.

Tables V to X show that the medium used (Medium A, Table V), is suitable for *B. subtilis* and that the spores can be counted accurately, the errors involved being no greater than the normal errors of random sampling. Having shown that satisfactory viable counts of *B. subtilis* spores could be performed if the spores are in the form of an even suspension, the next task was to examine the spray-dried powder containing these spores. Table XVIII shows two things. In the first place the mortality on drying is low and in the second place variations in the conditions of drying, e.g., in the temperature of the inlet air or the rate of flow of the liquid, have comparatively little effect on the percentage of organisms surviving. Thus one might expect to obtain an even distribution of spores in the resultant powder since local conditions at the jet, or variations in the length of time the powder is lodged in the machine, would not be expected greatly to alter the count of different portions of the powder. Further, in a previous paper it was shown that spores in dry powders were resistant to comparatively high temperatures. It has also been shown that the spores remain viable in the resultant powder over considerable periods of time and that there is little, if any, diminution in the viable count of the powder (Table XXVIII). That the expected even distribution of spores in the powder is in fact obtained is proved in Table XXI. The count variation from sample to sample of powder is shown statistically to be accounted for by the normal errors of random sampling. It is clear from the conclusions established that spray-dried powders containing *B. subtilis* spores are suitable for use

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in further experiments. Such experiments involving the exposure to antiseptics and heat of both the powder itself and the powder suspended in oil, are in progress in this department.

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3. It has been shown that if even suspensions of *B. subtilis* spores are spray-dried the viable organisms are evenly distributed in the resultant powder.

4. It has been shown that if even suspensions of *Bact. lactis aerogenes* are spray-dried the viable organisms are not evenly distributed in the powder as taken from the spray-drier, but that an even distribution can be obtained if the powder is thoroughly mixed by mechanical means.

5. The effect of storage on the viability of the organisms in peptone powders has been examined. The viable count of *B. subtilis* was found to undergo no significant diminution after 6 months' storage, whereas the viable count of *Bact. lactis aerogenes* fell rapidly, most of the organisms dying within the first few days.

6. It is concluded that spray-dried powders containing spores of *B. subtilis* are very suitable for examining the effects of environmental conditions on the spores. On the other hand, the use of powders containing organisms, such as *Bact. lactis aerogenes*, which show a high mortality on spray-drying, involves greater difficulties, which may be inherent in the problem.

It is a pleasure to express our thanks to Professor M. S. Bartlett for suggestions and advice concerning the statistical treatment of the results reported in this paper.

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DISCUSSION

Miss Winifred Keepe presented the paper.

MR. B. A. BULL (Nottingham), deputising for the Chairman, said that the work described was an extension of the valuable work of Dr. Bullock and his colleagues on spray drying. The authors seemed to have evolved a suitable technique for a complex subject.

DR. K. R. CAPPER (London) said that bacteria in dust were in an environment of low moisture content, and dust was a very probable

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT.

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source of infection. Apparently a greater number of cells developed from a medium of lower food content. It might be advisable to use a peptone medium containing yeast extract and not meat extract. With *B. subtilis* he had found greater or more consistent growth in liquid media containing yeast extract, and, in particular, in yeast autolysate dextrose media. The addition of such substances might not increase the number of cells which actually grew to produce colonies; but he would like to know whether the authors had tried yeast extract or any type of yeast media or the addition of trace elements. When cells were under favourable conditions certain parts of their enzyme systems were likely to be destroyed or damaged before others, and the composition of the medium used was then of considerable importance. He did not think that it was certain that the type of medium used in sterility tests was the best, or that the type of control using diluted 24-hour cultures was necessarily the best type of control. Cells which had been damaged by drying, for instance, might not grow, whereas fully viable cells would grow. The time of incubation was 60 hours, but spores were liable to become dormant and to develop in much longer periods; he believed that Dr. Davis had found that certain spores developed after some weeks. There was considerable difficulty in keeping solid cultures in aga media for that length of time, though there were techniques for doing this.

DR. I. MICHAELS (London) said that the authors by treating their results statistically had brought their work into line with that of other contributors in this field. Two important factors had been studied; the first was the behaviour of the sporing organism on a roll tube, and the second was the use of volume tests for accurate dilutions. The standard error of the mean of the three counts in Table III, Method (3), namely, 2.94, was well within the accepted limit of ± 5 per cent. The number of organisms on the tubes was rather high, and must have been responsible for much tedious work. The rating of volumes was far less troublesome than counting numbers of groups, but the choice of techniques depended largely on the number of organisms. For comparatively small numbers, such as the hundreds of thousands which the authors had employed, the degree of dilution was not high. However, when hundreds of millions of organisms were used, as when dealing with bactericidal activity of disinfectants, the degree of dilution was considerable, and a technique which did not allow a high dilution at each stage, coupled with the employment of small but accurately determinable quantities, would render the overall technique unmanageable.

MR. G. R. MILNE (Glasgow) asked what was the moisture content of the powders prepared by spray drying and stored, he thought, over phosphorus pentoxide, and what was the likely effect of small variations in this moisture content on the organisms in the powder. For dried

4. It has been shown that if even suspensions of *Bact. lactis aerogenes* are spray-dried the viable organisms are not evenly distributed in the powder as taken from the spray-drier, but that an even distribution can be obtained if the powder is thoroughly mixed by mechanical means.

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DR. K. R. CAPPER (London) said that bacteria in dust were in an environment of low moisture content, and dust was a very probable

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT. PART I

source of infection. Apparently a greater number of cells developed from a medium of lower food content. It might be advisable to use a peptone medium containing yeast extract and not meat extract. With *B. subtilis* he had found greater or more consistent growth in liquid media containing yeast extract, and, in particular, in yeast autolysate dextrose media. The addition of manganese sulphate tended to give a more consistent growth. The addition of such substances might not increase the number of cells which actually grew to produce colonies; but he would like to know whether the authors had tried yeast extract or any type of yeast media or the addition of trace elements. When cells were under favourable conditions certain parts of their enzyme systems were likely to be destroyed or damaged before others, and the composition of the medium used was then of considerable importance. He did not think that it was certain that the type of medium used in sterility tests was the best, or that the type of control using diluted 24-hour cultures was necessarily the best type of control. Cells which had been damaged by drying, for instance, might not grow, whereas fully viable cells would grow. The time of incubation was 60 hours, but spores were liable to become dormant and to develop in much longer periods; he believed that Dr. Davis had found that certain spores developed after some weeks. There was considerable difficulty in keeping solid cultures in agar media for that length of time, though there were techniques for doing this.

DR. I. MICHAELS (London) said that the authors by treating their results statistically had brought their work into line with that of other contributors in this field. Two important factors had been studied; the first was the behaviour of the sporing organism on a roll tube, and the second was the use of volume tests for accurate dilutions. The standard error of the mean of the three counts in Table III, Method (3), namely, 2.94, was well within the accepted limit of ± 5 per cent. The number of organisms on the tubes was rather high, and must have been responsible for much tedious work. The rating of volumes was far less troublesome than counting numbers of groups, but the choice of techniques depended largely on the number of organisms. For comparatively small numbers, such as the hundreds of thousands which the authors had employed, the degree of dilution was not high. However, when hundreds of millions of organisms were used, as when dealing with bactericidal activity of disinfectants, the degree of dilution was considerable, and a technique which did not allow a high dilution at each stage, coupled with the employment of small but accurately determinable quantities, would render the overall technique unmanageable.

MR. G. R. MILNE (Glasgow) asked what was the moisture content of the powders prepared by spray drying and stored, he thought, over phosphorus pentoxide, and what was the likely effect of small variations in this moisture content on the organisms in the powder. For dried

human plasma, prepared by a freeze drying process, what would be the effect of bad sealing, and perhaps bad storage, on the content of bacteria.

MR. R. MAXWELL SAVAGE (Barnet) asked whether any of the experiments described had been extended to the anærobic sporing organisms, because there was a very interesting ecological point involved. In an attempt which he made some years ago to prepare an artificial powder containing anærobic spores he found that they died off with extreme rapidity.

DR. K. BULLOCK, in reply, said that, although the authors had adopted statistical techniques in the present paper, they had not yet become firm and consistent worshippers of statistics; in fact, so far they had not used them, in the sense of coming to any conclusion as a result of their statistics which would not have been formed from a simple inspection of the figures. The statistical analysis, however, would be necessary later on, when they had to decide such matters as whether a count had not fallen at all or had fallen slightly. In any case the present paper was a preliminary one. He thanked Dr. Capper for his remarks on sensitive media. They know that some of the cells were bound to be damaged, but they had incubated for a long time and had tried as far as possible to take the matter into consideration. It was, however, something which would have to be carefully reconsidered at various stages as the research developed. The moisture content of the powders in question when dried over phosphorus pentoxide was fully described in a previous paper by Mr. Wright and himself, where they stored these powders, having exposed them to various aqueous pressures, and then recorded the fall or otherwise of the counts. They had not dealt with the anærobes, because the present type of spray dryer was not suitable; it would be necessary to spray in a current of nitrogen.

MISS KEEPE, replying to Dr. Michaels with regard to the number of organisms, said that they chose between 200 and 400 because that number had been reported by previous workers to be the most suitable number.

PROTEIN IN MALTED PREPARATIONS

By F. WOKES AND CHLOE KLATZKIN

From the Ovaltine Research Laboratories, King's Langley, Herts

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PREVIOUS communications^{1,2,3,4} from these laboratories have dealt with the general food value of malted preparations, particularly malted barley and malt extract, giving data on the content of B vitamins (aneurine, nicotinic acid and riboflavine), of carbohydrates and of "protcin." The present communication deals with the nature of the nitrogenous constituents included in the term "protein."

The nitrogenous constituents of malt extract have received poor recognition from official sources. The British Pharmacopœia, 1932, specified a minimum protein content of 4.5 per cent. based on 6.25 times total N₂, but the British Pharmaceutical Codex, 1934, attributed the nutritive action of malt extract to carbohydrates and B vitamins, ignoring the protein, and the United States Pharmacopœia XII also ignored protein as a constituent. Since the malt extract of U.S.P. XII also contained 10 per cent. of glycerin, giving a more readily fermentable product for which sterilised containers had to be prescribed, it was perhaps fortunate that it was excluded from the U.S.P. XIII. The 4.5 per cent protein minimum of the B.P. 1932, can be considerably exceeded in a good malt extract, as one of us (F.W.) showed in 1943. Nevertheless, the protein minimum in the B.P. 1948 has been lowered to 4.0 per cent.. A moderate diastatic value was introduced and then almost immediately withdrawn. Previous workers⁵ have recommended the use of malt extracts with very low diastatic values for malt and oil manufacture, but this would involve the loss of protein and B vitamins. We hope to show that the proteins of malt extract and of other malted preparations are of greater importance than these pharmaceutical divagations indicate.

METHODS

Total nitrogen (micro-method). A quantity of material containing about 3 mg. of N₂ was heated with 2 ml. of concentrated sulphuric acid and a trace of mercuric sulphate and 50 mg. potassium sulphate for half an hour after charring had been completed. The cooled solution was placed in a micro-Kjeldahl flask, 10 ml. of 40 per cent. sodium hydroxide solution and 1 ml. of 40 per cent. sodium sulphide solution were added and the contents of the flask were distilled with steam into 10 ml. of N/20 sulphuric acid. The latter was boiled to remove carbon dioxide and titrated against N/20 sodium hydroxide using methyl red as indicator. The difference between the reading and that given by a blank with reagents only represented the nitrogen from the sample. The micro method was employed to estimate minute amounts of nitrogenous constituents in germinated seeds of which only small quantities were available. It was controlled against ammonium sulphate and gave good agreement with the B.P. 1948 method.

Salt soluble nitrogen. The method of the Association of Official

Agricultural Chemists⁶ was used with 1 per cent. of sodium chloride instead of 5 per cent. sodium sulphate, to minimise bumping during concentration of solutions. This alteration made no difference to the results.

Non-protein nitrogen. The method of the Association of Official Agricultural Chemists⁶ was used.

Amino acids were estimated chemically by the methods described by Block and Bolling⁷. Some of these (e.g. for tryptophane) did not give very reliable results and our findings with them must be considered only tentative. However, with arginine, one of the most important amino-acids in our materials, we used Dubnoff's⁸ modification of Sakaguchi's method⁹, and obtained on a series of oats samples good agreement with results obtained at the Cereals Research Station, St. Albans, by Mr. J. G. Heathcote, who has had considerable experience of this problem, and to whom we are indebted for advice and help.

Aneurine was estimated fluorimetrically by the method¹⁰ previously described which has given good agreement with microbiological methods.

Nicotinic acid was estimated colorimetrically using either *para*-aminoacetophenone¹¹ or *para*-aminopropiophenone¹² as the aromatic amine. Both of these gave satisfactory agreement with microbiological assays.

Riboflavine was estimated fluorimetrically by a method¹³ giving good agreement with microbiological assays.

Diastatic index was estimated as previously described¹.

Trypsin inhibitor of soya and other foods was estimated by a modification of the method of Bowman¹⁴ using skimmed milk instead of casein as substrate, and following the process of digestion by formol titrations.

RESULTS

Differentiation of nitrogenous constituents. (a) *Total nitrogenous constituents.* The B.P. 1948 method estimates total nitrogen by the Kjeldahl method and multiplies the result by 6.25 to determine the protein content of malt extract. This involves two faulty assumptions—that the factor 6.25 is accurate for cereal proteins, and that the nitrogenous constituents are all protein. We have overcome both these difficulties by quoting our results as nitrogen instead of as protein content. On this basis Extract of Malt B.P. 1948 should contain at least 0.64 per cent. of total nitrogen. This seems quite a low limit. Our results on 37 samples from 13 manufacturers, summarised in Figure 1, show that only two samples fell significantly below this lower limit, but seven samples fell below the B.P. 1932 limit. Our own samples were all well above this higher limit. Confirmation was provided of our previous finding that a low total nitrogen is usually accompanied by a low diastatic value. Data on malted barleys also given in Figure 1 show a similar tendency for the total nitrogen content to fall with decrease in diastatic value. However, we think that the wide variation in the total nitrogen of malt extracts is probably due partly to differences in brewing conditions.

(b) *Protein nitrogen* (from albumin and globulin plus "less soluble" protein). Our results in Table I show that only 12 to 44 per cent. of the total nitrogen content of malt extract represents true protein, as distinct from protein digestion products.

PROTEIN IN MALTED PREPARATIONS

(c) *Salt-soluble nitrogen.* This figure includes nitrogen from albumins and globulins as well as from the breakdown products of these and other proteins, all of which are important in infant feeding. It therefore provides valuable information on the nutritive value of malted preparations. Our results (see Table I) show that it can vary significantly in different samples.

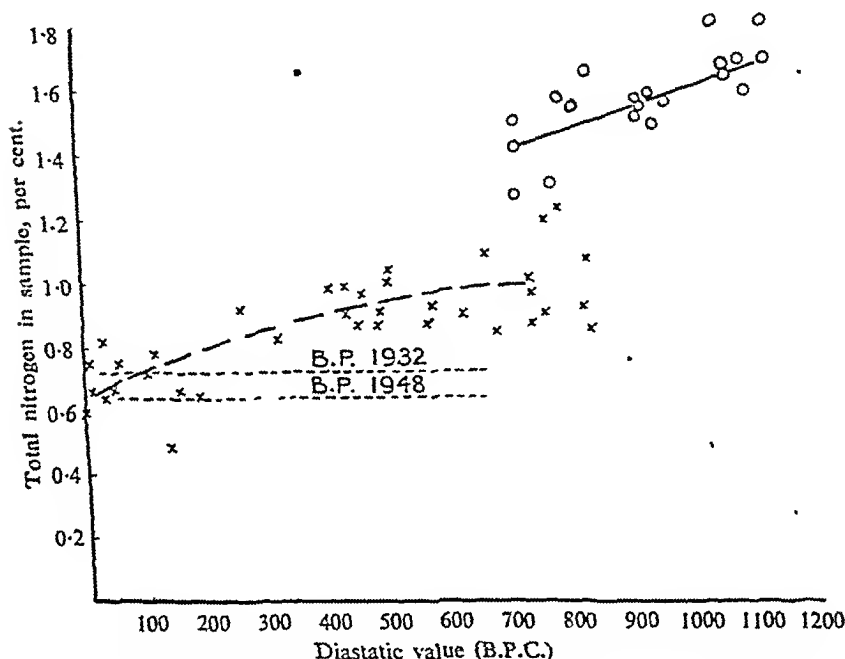


FIG. 1. Relation between diastatic value and total nitrogen content of malted barley (indicated thus \bigcirc — \bigcirc) and of malt extract (indicated thus X—X).

(d) *Non-protein nitrogen.* This figure includes the soluble breakdown products of the cereal proteins, with possible slight traces of asparagine and glutamine (which are important in plant metabolism¹⁵ but have yet

TABLE I
DIFFERENTIATION OF NITROGENOUS CONSTITUENTS IN MALT EXTRACT

Sample	Salt-soluble nitrogen	"Non protein" nitrogen	Albumin and globulin nitrogen	"Less soluble" nitrogen	Total nitrogen
3C	1.60	0.98	0.62	0.08	1.68
3B	1.47	0.98	0.49	0.13	1.60
3D	0.94	0.57	0.37	0.07	1.01
9B	1.10	0.87	0.23	0.03	1.13
9A	0.93	0.72	0.21	0.06	0.99
9C	0.93	0.72	0.21	0.05	0.98
5A	0.815	0.73	0.085	0.015	0.83
7A	0.74	0.56	0.18	0.08	0.82
8A	0.72	0.53	0.19	0.08	0.80
3A	0.51	0.40	0.11	0.03	0.54

d globulin nitrogen=salt-soluble-non-protein nitrogen.
rogen=total nitrogen-salt-soluble nitrogen.

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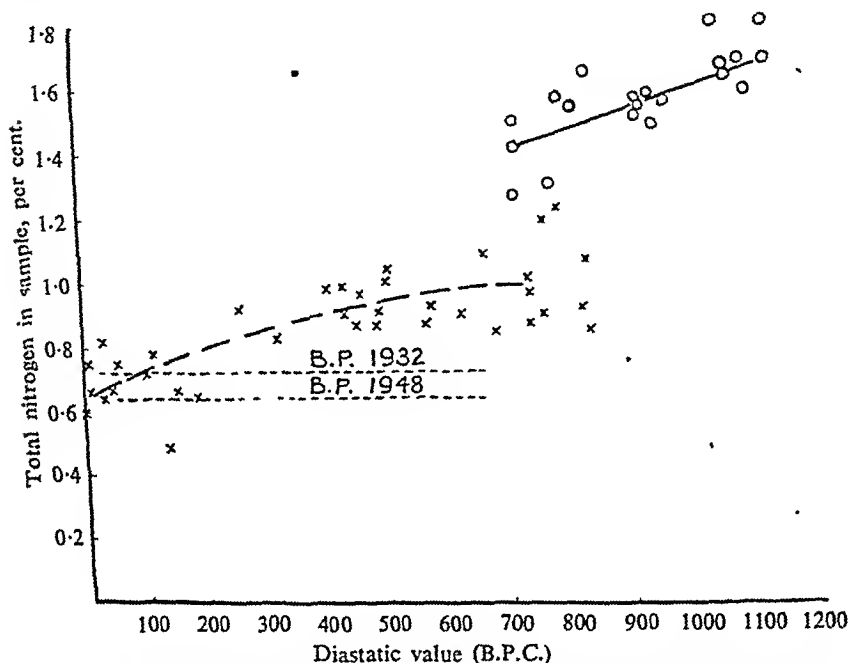


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5 A	0.815	0.73	0.085	0.015	0.83
7 A	0.74	0.56	0.18	0.08	0.82
8 A	0.72	0.53	0.19	0.08	0.80
3 A	0.51	0.40	0.11	0.03	0.54

Notes.—Albumin and globulin nitrogen=salt-soluble-non-protein nitrogen.
"Less soluble" nitrogen=total nitrogen-salt-soluble nitrogen.

to be established as significant in animal metabolism). The figure provides a useful measure of the extent of enzyme action during malting and brewing.

(e) "*Less soluble*" nitrogen. This figure represents the structural protein of plants, but would also include protein coagulated by heat during manufacturing processes. Little is yet known about its degree of availability to infants or its importance in infant nutrition. Our results in Table II show that it can vary widely in different samples, and we would suggest that preference be given to malted preparations in which the "*less soluble*" nitrogen has been reduced to a low level.

TABLE II
PERCENTAGE DISTRIBUTION OF NITROGENOUS CONSTITUENTS IN TOTAL NITROGEN IN MALT EXTRACT

Sample	Percentage of total nitrogen represented by nitrogen from		
	Albumin and globulin	Predigested protein	"Less soluble" protein
3 C	37	58	4.8
3 B	31	61	8.1
3 D	37	56	6.9
9 B	20	77	2.7
9 A	21	73	6.0
9 C	21	74	5.1
5 A	10	88	1.8
7 A	22	68	9.8
8 A	24	66	10.0
3 A	20	75	5.5

Protein value of malted preparations in infant feeding.

The experience of one of us (C.K.) shows that malted preparations are widely employed in London hospitals as dietary supplements for young children, especially those who are undernourished. Whilst some of the nutritive virtues of these malted preparations are doubtless due to their vitamin content, the presence of readily assimilated protein and carbohydrate must be of value. As long ago as 1865 Liebig¹⁶ showed by experiments on his breast-fed grandsons that a deficiency in the supply of human milk could be made good by "Malzsuppe," a malted infant food prepared by heating an aqueous extract of malted barley with wheat flour and milk. The milk provided about one-third of the total solids and two-thirds of the total protein. This led to the introduction of malt extract for infant feeding, and perhaps provided the inspiration for the Italian brewing chemist, Caprino, to devise during wartime emergencies milk substitutes based on malted cereals. In Italy oats are a staple cereal, and hence were used by Caprino, leading to the name "Maltavena." Ward Perkins¹⁷, a British Red Cross worker interested in the Italian experiments passed on the idea to U.N.R.R.A. in London and experiments were begun in England. Here oat flour was replaced by malted barley and wheat flour, and soya flour was added to improve the protein value, but the name "Maltavena" was still retained. A series of experimental batches were made in our laboratories, and tested clinically on babies and physiologically on rats. The clinical tests were

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not sufficiently comprehensive to show more than that babies could tolerate Maltavena. The physiological tests, carried out at Cambridge by Chick and Slack¹⁸, showed that some of the samples closely resembled milk in their growth-promoting properties.

In view of these physiological results it was decided to institute large scale clinical trials in Germany. We prepared some of the material used in these trials, sending over a ton to Germany during 1947. The results of these large-scale clinical trials have not yet been published. However, a few clinical tests of our materials have also been made in this country. Sufficient data is not yet available to permit publication of precise figures, but the indications are that malt and soya baby foods containing about 10 per cent. of "protein" (based on a total nitrogen content of 1.67 per cent.) are inadequate for satisfactory growth. Better results have been obtained with these foods containing about 12 per cent. of "protein" (based on a total nitrogen content of 2.08 per cent.). At this point, however, a new factor came into view, viz. the occurrence in raw soya of a trypsin inhibitor¹⁴ which might affect the digestibility of the Maltavena *in vivo* and thus its availability for growth promotion. On applying *in vitro* tests to the soya used as raw material we found that it contained the trypsin inhibitor, which could be removed by acid extraction and destroyed by autoclaving or enzyme action. Moreover, the Maltavena which we had sent to Germany for the clinical trials also contained the inhibitor. Our method of testing for the inhibitor did not permit its precise evaluation using milk as substrate. Such evaluation was effected by Dr. Borchers of Nebraska University, a leading American investigator in this field, who very kindly examined some of our Maltavena samples and confirmed that the inhibitor was present (see Table III).

TABLE III
TRYPSIN INHIBITOR IN SOYA AND IN MALT AND SOYA FOOD

Materials examined	Inhibitor found by	
	Borchers	Ovaltine Research Laboratories
Soya beans		
Soya meal, defatted		+++
" " full fat...		+++
" " heated dry 1 hr. at 100°C.		+++
" " autoclaved 1 hr. at 115°C.		++
" " digested with papain		0
Malt and soya food 1945*	100	0
" " 1947	0	trace
	5	
	2	

Notes—
* Tested physiologically for growth promoting properties by Chick and Slack¹⁸.
Results show relative amounts of inhibitor found using as substrates haemoglobin (Borchers, private communication) and milk (O.R.L.). Dr. Borchers does not consider that his results indicate the presence of any significant amount of the inhibitor in the two samples.

It was then decided to make further samples in which the inhibitor would be destroyed by autoclaving. Unfortunately this treatment affected the thermoplastic nature of the product, so that vacuum drying became impracticable, and before this difficulty could be overcome the opportunity had passed for taking part in the further German trials. *Vitamin B in Malt and Soya Baby Foods.* Before applying growth

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results to measure the relative protein values of different samples of malt and soya foods it is perhaps advisable to consider their content of B vitamins. In contrast with vitamins A, C and D, these were not supplied as supplements in all the clinical trials. Hence, if they were deficient in any of the samples this might have affected the growth results and thus confused the issue. Jeans and Marriott¹⁹ in their well-known textbook on Infant Nutrition, state that when infants are fed on milk substitutes based on soya the diet will need supplementing with B vitamins. Table IV shows the content of aneurine, nicotinic acid and riboflavine as

TABLE IV
COMPOSITION OF MALT AND SOYA BABY FOOD

No. of samples examined	2 *
Total solids per cent. w/v	95.0
Composition of total solids:—							
Total nitrogen per cent.	1.68
carbohydrate	84.2
fat	2.6
ash	2.6
Ca	mg./100 g.	559 †
Fe	6
aneurine	μg./g.	4.6
nicotinic acid	100 ‡
riboflavine	2.3
percentage of total calories from total nitrogen	10
„ of total calories from soluble nitrogen	6.0

Notes—

* From over a ton of dried product which had been thoroughly mixed to ensure uniformity. Dr. R. G. Booth's results on the samples were in good agreement with ours.

† Confirmed by microbiological assays by Dr. F. W. Norris. In calculating percentage of total calories from total nitrogen and from soluble nitrogen allowance was made for lower nitrogen factors of vegetable protein.

‡ Most of this comes from soluble calcium salts used in the formula.

μg./g. in the "10 per cent." sample. These data have now to be compared with the actual requirements of babies, and the amounts in human milk, which are given in Table V on the same basis of μg./g. total solids of diet and of human milk respectively. The figures for aneurine and nicotinic acid in human milk are considerably lower than the suggested requirements, and it seems safer to assume that the latter are more reliable. The nicotinic acid figure especially has been queried by later workers²⁰. On this requirement basis our samples probably contained

TABLE V
VITAMIN B VALUE OF MALT AND SOYA FOOD AND ITS RAW MATERIALS

Material	content as μg./g. total solids		
	aneurine	nicotinic acid	riboflavine
Malt and soya food "10 per cent. protein"*	4.6	100	3
Human milk, solids of	2.4†	16	2-7.5§
Requirements in diet of baby†	5.5	55	8
Malt extract solids*	2.5 to 4.5	100 to 150	2 to 4.5
Per cent. from raw materials*—			
Malt extract	75	95	88
Soya	19	2	7
Wheat	6	3	3-4

* Results obtained in Ovaltine Research Laboratories. † Calculated from data from U.S.A. National Research Council ²¹. ‡ Calculated from data by Knott, Kleiger and Schlutz ²². § Calculated from data of Marriott and Jeans ²³.

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enough aneurine, and certainly supplied enough nicotinic acid. They may, however, have been deficient in riboflavine. Later samples have been fortified with this vitamin.

Table V shows the proportion of these B vitamins supplied by the different raw materials. It will be seen that by far the greater part of the vitamins comes from the malt extract. Since commercial malt extracts vary widely in their content of B vitamins, stress is laid on the importance of ensuring that this is as high as possible in any malt extract used in the preparation of malt and soya baby foods. This applies particularly to riboflavine, of which soya is rather a poor source. By taking sufficient care in the malting and brewing processes it is possible to obtain malt extracts which are considerably better sources of this vitamin than soya is.

The nicotinic acid content of malt and soya foods made from malted barley should not present much difficulty, judging by the fairly narrow range in commercial malt extracts. If, however, malted barley were replaced by malted oats, as Caprino originally employed, the nicotinic acid content might be more critical. Unmalted oats contain only about one-tenth as much of this vitamin as unmalted barley²⁴ and in contrast with the latter, the nicotinic acid content can at least double during germination, but will still be only one-fifth of that in malted barley. A similar objection applies to the use of maize in baby foods.

Another point to be considered is the possible loss of B vitamins during removal or destruction of the trypsin inhibitor in soya. Our experiments indicated that the destruction of the inhibitor by autoclaving would involve losing 10 to 20 per cent. of the aneurine in the soya. Table V shows that this would be only 2 to 4 per cent. of the total aneurine in the "10 per cent." sample. This loss is insignificant. Much greater losses of aneurine can occur by heat treatment during the preparation of soya flour from soya beans. Removal of the inhibitor by extraction with very dilute acid as used by American workers²⁵ removed much more aneurine and also a good deal of nicotinic acid and riboflavine.

The trypsin inhibitor of soya may also be destroyed by dry heating at a sufficiently high temperature. Recent work²⁶ shows that such dry heating may, if carried on long enough (say five hours), cause the protein to be less rapidly digested by the enzymes of the gastro-intestinal tract. The amino-acid lysine seems to be particularly affected.

American workers²⁶ suggest that with optimal processing conditions 50 to 65 per cent. of the protein in soya should be extractable with water. American samples of soya vary widely in their content of water-soluble protein, some containing practically none. In the nitrogenous constituents of the soya we used in manufacturing our samples about 20 per cent. was water-soluble, which is higher than is indicated by the protein figures for some American samples but well below the optimum. Table II showed that in commercial malt extracts at least 90 per cent. of the nitrogenous constituents are soluble. Since this is a much higher proportion than the corresponding figure for soya, increase in the proportion of the latter in Maltavena would be expected to lower the proportion of soluble in total nitrogenous constituents.

about 8 per cent., after which it levels out between 7 and 8 per cent. on a calorie basis. Since milk substitutes are hardly ever given until after the first few weeks of lactation it seems fair to compare them with human milk containing about 8 per cent. of protein (on a calorie basis). This

TABLE VIII

COMPARISON OF MALT AND SOYA FOOD WITH HUMAN MILK AS A SOURCE OF ESSENTIAL AMINO ACIDS

Amino-acid	" 10 per cent." sample	" 12 per cent." sample
Arginine	106	138
Lysine	80	102
Tyrosine	68	90
Tryptophane	93	114
Phenylalanine	104	129
Histidine	96	117
Cystine + Methionine	54	62
Threonine	101	123
Leucine	76	93
iso-Leucine	75	90
Valine	56	66

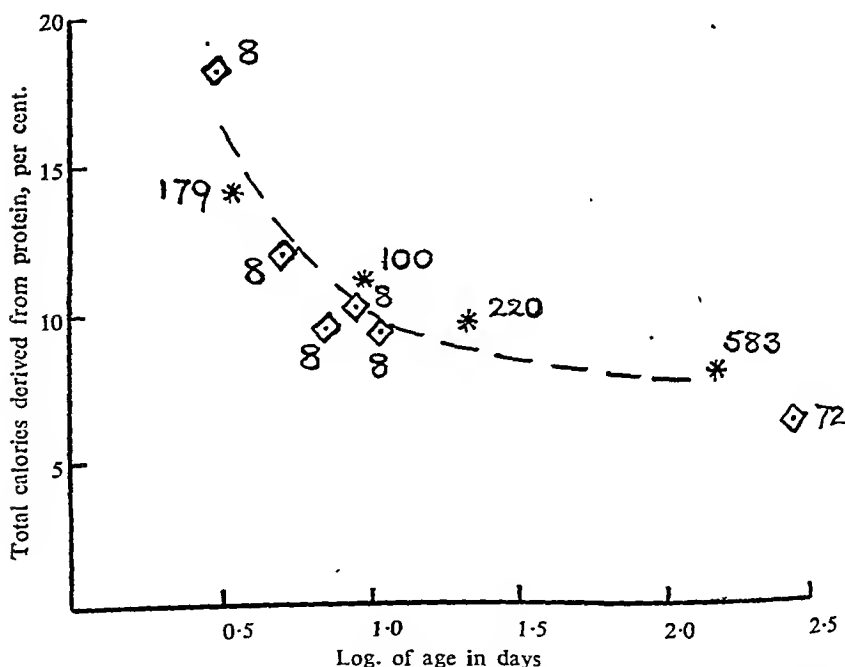


FIG. 2. Relation between protein content of human milk (on calorie basis) and stage of lactation plotted from data published by Gardner and Fox²⁹ (indicated thus *) and Hammett³⁰ (indicated thus ◇). Figures against each point refer to number of samples analysed, the majority of these representing all but the first month of lactation gave an average of 8.0.

suggestion is in agreement with Jean and Marriott's¹⁹ findings. An average value of 8 per cent. would imply that in Table VII the figures for

PROTEIN IN MALTED PREPARATIONS

the "10 per cent." sample could be multiplied by 1.25 and those for the 12 per cent. sample by 1.5 to obtain their amino acid values as compared with human milk. Table VIII shows the effect of doing this. In the 12 per cent. sample all the amino acid values have been brought close to those of human milk, except those for valine, whose importance is perhaps not so great, and for cystine+methionine, for which figures quoted are almost certainly too low. On this basis the protein value of human milk should be definitely superior to that of the 10 per cent. sample, but perhaps not greatly superior to that of the 12 per cent. sample. Moreover, it should be possible to prepare from cows' milk by careful adjustment of the proportion of curd and whey proteins a baby food containing about 8 per cent. of protein (all from milk) which would have a protein value similar to that of human milk. The clinical data so far obtained are in line with these assumptions.

SUMMARY

1. The total nitrogen content of malt extract may range from 0.2 to 1.2 per cent. but is usually well above 0.64 per cent. which corresponds to the B.P. 1948 minimum content of 4 per cent. (total nitrogen $\times 6.25$).
2. Lower diastatic values are usually associated with lower total nitrogen contents, the tendency being observed with malted barley as well as with malt extract.
3. Of the total nitrogen of malt extract only 12 to 44 per cent. is true protein nitrogen. The greater part represents breakdown products of protein, largely formed during malting and brewing.
4. The "less soluble" nitrogen of malt extract may range from 1.8 to 10 per cent. of the total nitrogen, and should be reduced to as low a level as possible in malted preparations.
5. In baby foods prepared from malt extract, wheat flour and soya flour sufficient aneurine and nicotinic acid may be derived from the raw materials if care is taken to use malt extract of high vitamin B content. Fortification with riboflavine may be necessary. If the diet is adequately supplemented by vitamins A, C and D the growth-promoting effect on babies may provide a measure of the protein value of the diet.

We are indebted to Dr. F. W. Norris for microbiological assays, to Dr. R. G. Booth and to Mr. J. G. Heathcote for checking at the Cereals Research Station some of our protein analyses, to Miss Hazel Williams for fluorimetric estimations of aneurine, to Miss Janet Horsford for technical assistance, and to Dr. H. Chick and her colleagues for advice and generous comments on our work.

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FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS

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IN a previous communication¹ we described fluorimetric and microbiological assays of riboflavin in barley, malted barley and malt extract. Whilst the two assay methods gave the same general picture for increases in content of this vitamin during malting and brewing, certain discrepancies were encountered between the results given by the two methods. On 4 samples of barley and malted barley, the fluorimetric method gave lower results (72 to 89 per cent. of the microbiological result), the deviation on 3 of the samples being significant. On 4 samples of malt extract the fluorimetric result did not deviate significantly from the microbiological result. On a fifth sample of malt extract the mean fluorimetric result was 231 per cent. of the mean microbiological result, a highly significant difference. Extensive investigations have been carried out in our laboratories to explain these discrepancies, and show how they may be overcome by improvements in fluorimetric and chromatographic technique.

Elvidge² reported good recoveries (84 to 105 per cent.) of this vitamin from 5 more potent pharmaceutical preparations (an elixir, capsules and tablets) in which he estimated it fluorimetrically or spectrophotometrically. A detailed study of the spectrophotometric method was made in our laboratories³. Optical densities were recommended to be read at 267 or 375 m μ as well as at the maximum of 444 to 445 m μ recommended by Elvidge. The importance of controlling the pH was emphasised, and experiments described on the development of lumiflavin through the action of light on alkaline solutions of riboflavin. The possible interference of lumiflavin fluorescence has been one of the factors we have had to investigate in the fluorimetric work described below.

MICROBIOLOGICAL ASSAYS

The method as laid down by the Analytical Methods Sub-Committee of the Society of Public Analysts⁴ was almost exactly followed. It has been described with comments in a previous paper¹. Continued experience with the method has shown that *Lactobacillus helveticus* is not on the whole so easy to work with as, for example, *Lactobacillus arabinosus*, which is used for the assay of nicotinic acid and biotin. Difficulties are sometimes experienced in maintaining sub-cultures, and it would seem that the organism requires nutritives whose nature is not necessarily exactly known, but which are present in some natural products. This is further indicated by the observation in recent months that there is a tendency to obtain less satisfactory linearity in the standard curve than in the curves for the assay samples.

The effect of the presence of fats and fatty acids in samples and

extracts undergoing assay has long been observed, although completely satisfactory explanations are not as yet forthcoming (see, for example, Kodicek and Worden⁵, and Norris and Lynes⁶). The occurrence of fats as such in materials for assay need present little difficulty since the sample may be quantitatively pre-extracted with light petroleum in a Soxhlet extractor. The assay is then performed on the fat-free product, and the vitamin content calculated back to the original material. Where the fat or fatty acids occur in lipin combinations, the position is not so simple. Ordinary extraction as above removes little or none of the lipin, and resort to more drastic treatment involves loss of all or part of the vitamin. In these cases it has usually been satisfactory to extract the hydrolysed extracts with light petroleum or, better, ether.

FLUORIMETRIC ASSAYS

We previously employed a modification of the fluorimetric methods of Rubin *et al.*⁷ and of Hoffer *et al.*⁸ kindly given us by Dr. A. J. Amos, Secretary of the Chemical Panel of the Vitamin Sub-Committee of the Society of Public Analysts. Whilst this method as adapted for use with the Spekker fluorimeter gave reasonable agreement with microbiological assays on some foods fairly rich in the vitamin and relatively free from interfering substances, the Panel members did not find it satisfactory for malted preparations and certain other foods in which more of these interfering substances were present. The final conclusion of the Panel was that this method, although showing distinct promise, was not capable of general application until a more sensitive fluorimeter giving greater reproducibility was available. The Panel devised a deflection instrument of the Cohen type, which provided about 4 times the sensitivity of the old Spekker Fluorimeter, but did not find this to be satisfactory. Our own investigation of this instrument shows it to be unsatisfactory for riboflavine assays because of its high instrumental blank (see Table I).

TABLE I
PERFORMANCE OF DIFFERENT FLUORIMETERS IN RIBOFLAVINE ASSAYS

Type of fluorimeter	Relative sensitivity	Instrumental blank as per cent of F/1
Old Spekker	1	3
New "	7	10 to 15
Cohen type	3 to 4	15
Electronic	63 to 73	1 to 2

Notes—Relative sensitivities measured by deflections produced with F/1 using maximum sensitivity of each fluorimeter and the set-up of filters and cuvettes normally used in assays. Results given in comparison with Old Spekker as unity.

Instrumental blank determined with cuvette filled with distilled water and the usual set up of filters. Cohen type fluorimeter was prototype circulated to members of the Chemical Panel for critical examination, and was constructed to take test tubes instead of cuvettes.

Electronic fluorimeter used with photomultiplier at 78 volts per stage.

Attempts have been made to overcome the difficulty by developing a more sensitive fluorimeter which would permit the use of more selective filters and thus eliminate non-specific fluorescence in other parts of the spectrum. A prototype of the new Spekker fluorimeter (Hilger and

Watts) was found to possess still greater sensitivity, but its instrumental blank was still rather high. This blank has since been considerably reduced by improvements in the cuvette housing and by the use of black glass lids for the cuvettes. The improved instrument may, in our opinion, provide valuable service in riboflavine assays, especially if it is modified as we have recommended to take the new electron photomultipliers. We have been experimenting for over 2 years on the use of the latter to replace barrier layer photocells, and have constructed what is probably the most sensitive fluorimeter so far described in this country. Full details of this electronic fluorimeter were given to the Society of Public Analysts*. It employs as photoelectric detector either the RCA 1.P.21 or RCA 1.P.28 photo-tube or suitable British equivalents with a specially designed power pack and stable galvanometer. Table I shows that the sensitivity with the photomultiplier at 78 volts per stage is 63 to 73 times that of the old Spekker fluorimeter, and the instrumental blank has been brought quite low. It has given satisfaction during over a year's constant use, many of the results in this paper having been obtained with it.

FLUORIMETRIC METHOD

All chemicals to be of Reagent or similar quality. Pyridine to be freshly redistilled.

Extraction and hydrolysis. Weigh a sample containing about 25 to 35 $\mu\text{g.}$ of riboflavine. Add 50 ml. of 50 per cent. v/v concentrated hydrochloric acid and heat in a boiling water bath for 20 minutes. Cool. Make up to 100 ml. with distilled water. Filter.

Oxidation. To 30 ml. of extract adjusted to pH 4 to 4.5 with saturated solution of sodium acetate, add 1 ml. of 4 per cent. potassium permanganate solution. If the pink colour does not persist for 1 minute, add further 1 ml. quantities of permanganate solution until it does. Remove excess of permanganate by adding hydrogen peroxide (3 per cent.) drop by drop. Adjust the pH to 4.5. Add sufficient pyridine to bring the concentration of pyridine to 1 per cent.

Adsorption and elution. Prepare an adsorption column of special fuller's earth 8 to 10 cm. long.* Wash the column with about 25 ml. of 2 per cent. acetic acid solution, followed by 15 ml. of water. Pour the whole of the oxidised extract through the column. Wash the column with 1 per cent. pyridine, examining in ultra-violet light, until the yellow band due to riboflavine is clearly defined on the column. Elute with solvent (20 per cent. pyridine in 2 per cent. acetic acid), collecting the yellow fluorescent eluate. Dilute to 50 ml. with solvent.

Fluorimetry. Dilute a suitable aliquot of eluate with solvent to produce 25 ml. dilution containing about 0.15 $\mu\text{g.}$ riboflavine/ml. (= U). To another equal aliquot of eluate add 2 ml. of riboflavine standard (0.5 $\mu\text{g./ml.}$) in solvent and dilute with solvent to 25 ml. (= UR) Take 5 ml. of riboflavine standard (0.5 $\mu\text{g./ml.}$) and dilute to 25 ml. (= S).

* Florisil brand 60/100 mesh (Wilkens-Anderson, Co., Chicago) has been found suitable.

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FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE

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TABLE II

LINEARITY OF RESPONSE OF SPEKKER FLUORIMETER USING R.C.A.I.P.28 ELECTRON PHOTOMULTIPLIER

Calibration with riboflavine solutions at pH 5.7 matched against fluorescent standard F/4 (1 µg/ml) using Wratten 39 and H503 primary filters and Chance's orange OY2 secondary filter.

Concentration of riboflavine µg/ml	Mean density against F/4	Antilog density (AD)	$\frac{1}{AD}$	Fluorescence as percentage of F/4	Net F	$\frac{F}{C}$	Percentage deviation of F/C from mean
1	2	3	4	5	6	7	8
Fluorescein at zero							
$\frac{1}{2}$	387	2.438	—	243.8	237	237	0.2
$\frac{1}{4}$	049	1.248	—	125.8	119	238	0.7
Riboflavine at zero							
$\frac{1}{2}$	188	1.542	649	64.9	58.2	233	1.5
$\frac{1}{4}$	436	2.729	366	36.6	29.9	239	1.1
1/16	665	4.624	216	21.6	14.9	238	0.7
1/32	855	7.161	140	14.0	7.3	234	1.1
Solvent blank	1.175	14.96	067	6.7	—	—	—

Net F calculated by deducting solvent blank from gross fluorescence

greatest deviation is 1.5 per cent. and the mean deviation 0.9 per cent. In further calibration experiments the mean deviation was 0.4 and 0.2 per cent. (see Table III). This compares favourably with a mean deviation

TABLE III

EFFECT OF PRIMARY FILTERS ON DEGREE OF LINEARITY, SENSITIVITY AND INSTRUMENTAL BLANK OF ELECTRONIC FLUORIMETER

Primary filter	Relative sensitivity as F/C ratio	Solvent blank as percentage of F/4	Sensitivity solvent blank	Average percentage deviation from linearity
Woods	6.67	18.3	0.37	0.4
Wratten 39	2.37	6.7	0.35	0.7
" 47	1.82	3.6	0.51	0.2

tion of 0.7 per cent. in the calibration data for 4 fluorescein solutions ranging from 1.25 to 6 µg./ml. published by Cohen¹¹ in 1935, and not unsatisfactorily with the mean deviation of about 0.2 per cent. in data published on 5 fluorescein solutions by Umberger and La Mer¹² in 1945, especially considering that fluorescein solutions are much more stable than riboflavine solutions.

Choice of primary and secondary filters. Table III also shows the effect of using different primary filters, and thus varying the nature of the incident light beam, with the object of reducing the magnitude of the solvent blank, which influences the deviation from linearity. When the Wood's glass filter supplied with Spekker fluorimeters was replaced

Measure the fluorescence of U, UR, S and solvent only (= SB) against F/4 (0.25 $\mu\text{g.}$ fluorescein/ml. in phosphate buffer pH 7) separately in the same cuvette, using as primary filter Wratten 47 protected by H503 or other suitable heat-resisting filter, and as secondary filter Chance's orange OY2 or other suitable filter.

Calculation. Convert all densities into antilogarithms. Take reciprocals of antilog. densities for solutions weaker than the F/4 standard. Let the results be U, UR, S and SB. Subtract SB from U, UR and S to obtain net fluorescence for each. Then, assuming that the net fluorescence is proportional to the concentration

$$\text{concentration of riboflavin in the U dilution} = \frac{(S-SB) \times \text{concentration of riboflavin in dilution of standard}}{U-SB}$$

$$\text{concentration of riboflavin in the UR dilution} = \frac{(S-SB) \times \text{concentration of riboflavin in dilution of standard}}{UR-SB}$$

Calculate the concentration of riboflavin in the sample using the data for weight of sample taken and volume of aliquot of eluate taken. The result can be corrected for percentage recovery of the riboflavin added to UR by multiplying by $\frac{100}{\text{percentage recovery}}$.

Calibration of Fluorimeter. The usual method of calibrating a fluorimeter is to measure the fluorescence of a series of solutions, of which the strongest has 4 to 8 times the concentration of the weakest. If the fluorescence plotted against the concentration gives a reasonably straight line, the linearity of response is considered satisfactory over the given concentration range, which may be rather narrow. Thus in a recent paper¹⁰ a calibration curve for riboflavin was given based only on 4 solutions ranging from 0.06 to 0.24 $\mu\text{g./ml.}$

Our experience of riboflavin assays has led us to believe that calibration should be carried out over a much wider concentration range, including especially more dilute solutions to permit accurate evaluation of the blank. Moreover, we find it preferable to evaluate the linearity of response not visually from a calibration curve, but mathematically by calculating the ratio of net fluorescence to concentration of riboflavin, which should remain constant if the response is strictly linear. Table II gives details of one of our calibration experiments with the electronic fluorimeter. Column 2 in the Table gives the mean densities of a series of riboflavin solutions ranging from 1 down to 1/32 $\mu\text{g./ml.}$ (approximately 0.03 $\mu\text{g./ml.}$) as compared with fluorescein standard F/4. Column 3 gives the antilogs. of these densities and Column 4 the reciprocals of these antilogs. for the more dilute riboflavin solutions of which the fluorescence was weaker than that of the fluorescein standard, so that the zero was set on the riboflavin. Column 5 gives the gross fluorescence of each riboflavin solution as percentage of that of the fluorescein standard, and Column 6 gives the corresponding *net* fluorescence, calculated by deducting from the gross fluorescence the

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE

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Fluorescein at zero:—							
1357	2.438	—	243.8	237	237	0.2
1/2049	1.258	—	125.8	119	238	0.7
Riboflavine at zero:—							
1188	1.542	.649	64.9	58.2	233	1.5
1/2436	2.729	.366	36.6	29.9	239	1.1
1/16665	4.624	.216	21.6	14.9	238	0.7
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by a Wratten 39 filter (placed behind the H503 heat-resisting filter used with the Wood's) the solvent blank was reduced by about two-thirds, but the relative sensitivity was also reduced in similar degree, so that the sensitivity/solvent blank ratio was scarcely altered. The mean percentage deviation from linearity was increased from 0.4 to 0.7, so there did not appear to be any advantage in using the Wratten 39 as primary filter. With the Wratten 47 filter the decrease in the solvent blank was greater than the decrease in sensitivity, the sensitivity/solvent blank ratio being raised to 0.51. Moreover, the mean percentage deviation from linearity was reduced to the low figure of 0.2. Further calibration experiments with pure riboflavin solutions confirmed that the Wratten 47 filter gave better results than Wood's glass, and did not show the Wratten 47A filter to possess any distinct advantage over the Wratten 47. This justifies our choice of the Wratten 47 as primary filter. Figure 1 compares the transmission curves of these filters with the absorption and fluorescence spectra of riboflavin. If the fluorescence of riboflavin were due mainly to light absorbed above 400 $m\mu$, as previous evidence³ suggests, then the Wratten 39 or 47 filters should give higher results than the Wood's glass filter, which transmits practically nothing between 400 and 700 $m\mu$. In fact, however, the Wood's glass gives similar results to the Wratten 39 or 47 as primary filter, suggesting that some of the fluorescence may be due to light absorbed below 400 $m\mu$. (We have encountered some variation between different samples of filters. The data given in Figure 1 were obtained on the actual filters used in our assays.)

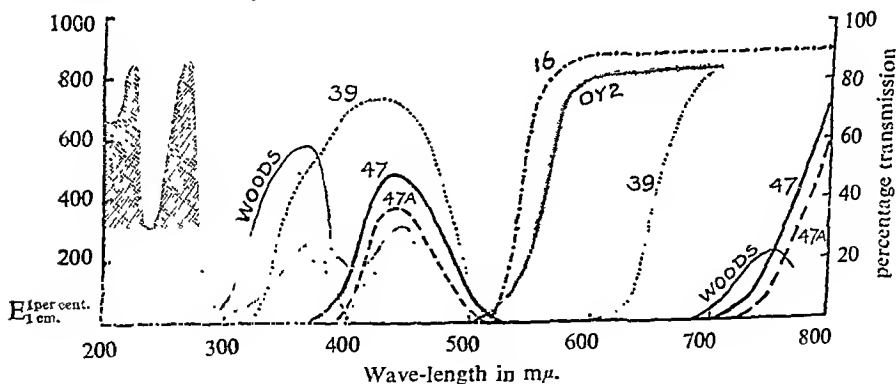


FIG. 1. Transmission curves of primary (Wood's, Wratten 39, 47, 47A) and secondary (Wratten 16, Chance's OY2) filters used in riboflavin fluorimetric assays, as compared with absorption and fluorescence spectra of the vitamin. The heavily shaded area between 200 and 500 $m\mu$ represents absorption spectrum under the given experimental conditions, and the lightly shaded area between 500 and 620 $m\mu$ indicates approximate position of fluorescence spectrum with maximum at about 570 $m\mu$.

Turning to secondary filters, these should transmit radiation corresponding to the fluorescence spectrum of riboflavin but exclude other radiation, either from non-specific fluorescence or from exciting light reflected from the lid and sides of the cuvette¹³. Until the fluorescence

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE

spectra of the interfering substances have been determined it is not possible to give a completely satisfactory theoretical basis for the selection of the secondary filters to be used in assays on malted preparations. However, since these preparations can exhibit marked non-specific blue fluorescence, it seems desirable to use a secondary filter which does not transmit blue light. Theoretically this requirement is fulfilled either by the Chance's OY2 orange filter, which we have mainly used during the last 4 years, or by the Wratten 16 which Kodicek and Wang recently recommended. In practice the exclusion of the blue fluorescence is not complete. For exclusion of reflected exciting light either the OY2 or the Wratten 16 filter is unsuitable for use with the Wratten 39 as primary filter, since the latter lets through light between 600 and 700 m μ , which is also transmitted by these secondary filters. This disadvantage can be overcome by using Wratten 47 or 47A as primary filter. The combinations of 47 primary with OY2 secondary, and of 47A primary with 16 secondary filters seem on a theoretical basis to be the best so far devised, and have given in our hands as good analytical results as any other combination of British or American filters we have tested, including the Lumetron 575. However, even the best filters may not provide effective correction when there is a considerable amount of interfering substance present. Table IV shows the effect of using different filter

TABLE IV

EFFECT OF DIFFERENT FILTER COMBINATIONS ON INTERFERENCE OF NON-SPECIFIC BLUE FLUORESCENCE IN MALTED PREPARATIONS

Filters		Net deflections (cm) given by		Percentage interference of blue fluorescence
Primary	Secondary	Blue non-specific	Riboflavine	
Wood's	OY2	3.35	14.7	23
39	"	17.2	72.4	24
47	"	5.7	26.3	22
47A	"	2.6	11.3	23
Wood's	16	4.9	22.5	22
39	"	25.0	127.0	28
47	"	8.2	43.0	19
47A	"	3.5	18.1	19
47A cal. . . .	"	3.4	15.9	21

Note.—Each of the above primary filters was protected by H503 filter, except the 47A Cal. where the H503 was replaced by a Calorex heat resisting filter. The numbers of filters refer to Wratten gelatin filters, except OY2 which is a Chance's glass filter.

combinations on a mixture of riboflavine with some of the non-specific blue fluorescent material obtained from malted preparations as described below. Using OY2 as secondary filter, no appreciable improvement in results was obtained by replacing the Wood's glass by 39, 47 or 47A primary filters. When Wratten 16 was used as secondary filter, the use of Wratten 39 as primary filter led to less satisfactory results.

Destruction of riboflavine to provide blanks. Various procedures have

sample needed only 1 ml. of the 4 per cent. permanganate per aliquot to give satisfactory results, and 3 ml. was far too much, a malted food sample needed 9 ml. per aliquot. Some of our old malt extracts required much more than 9 ml. Table VI shows that when too little permanganate has been used, the results can be considerably improved by using more specific filters to eliminate interfering fluorescence. The results in the Table were obtained using Florisil to remove interfering substances as described in our method above and show that it is equally essential to use the right amount of permanganate. If this is done, the choice of more selective filter combinations becomes less important.

(c) *Phase separation.* Our aqueous extracts were shaken with various immiscible organic solvents under the ultra-violet lamp to detect whether separation of the riboflavine fluorescence from non-specific fluorescence was being effected. With amyl alcohol, chloroform, ether and light petroleum, slight separation occurred, but could not be made complete. With benzene, diacetone alcohol and isobutyl alcohol no separation was detected.

(d) *Chromatographic separation.* Elvidge², using the Connor and Straub¹⁴ method, examined a number of grades of fuller's earth and selected P.A. of the Fuller's Earth Union as the most suitable. We tested a sample of this grade, but did not find it to give satisfactory purification of our materials. Other samples of fuller's earth, also of alumina and of Decalso, likewise proved unsatisfactory. We then tried Florisil, which has been largely employed by American workers, and found that it removed some, but not all, of the non-specific fluorescence when used as they recommend¹⁵. Observations under the ultra-violet lamp showed that riboflavine is adsorbed more strongly than the interfering substances. After numerous trials we found that elution with 1 per cent. aqueous pyridine could separate most of the interfering substances, the procedure being controlled under the lamp. 2 per cent. pyridine was seen to elute riboflavine almost as rapidly as it eluted the interfering substances, so that complete separation could not be effected. We found that the adsorption of riboflavine on Florisil was equally effective between pH 1 and pH 6. Contrary to American workers¹⁵, we did not find the adsorbed riboflavine to be removed by washing the column with large volumes of water. Elution with 20 per cent. pyridine in 2 per cent. acetic acid gave recoveries ranging from 90 to 100 per cent.

Riboflavine in malt extract. On studying the history of the 5 malt extracts for which fluorimetric and microbiological results were given in our paper¹, we found that the extract on which the fluorimetric method gave a much higher result than the microbiological method was in fact an old one. This led us to compare the fluorimetric and microbiological results on a series of malt extracts of different known ages, all stored at room temperature. The results plotted in Figure 2 showed a definite tendency for the fluorimetric/microbiological ratio to increase as the extracts become older. Assuming that a difference of 20 per

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE

cent. between the two methods was significant, then in 8 extracts less than 2 months old, not one showed a significant difference between the two methods, in 8 extracts 40 to 130 weeks old, 7 gave a fluorimetric result significantly higher than the microbiological result, the average fluorimetric/microbiological ratio being 1.78 and in 7 extracts $3\frac{1}{2}$ to $4\frac{1}{2}$ years old the fluorimetric result was always significantly higher than the microbiological result and the average fluorimetric/microbiological ratio was 2.64. (The fluorimetric result on one of this last 7 was too high to include in Figure 2.)

We confirmed these findings by assaying fluorimetrically and microbiologically at intervals the riboflavine in malt extracts stored under known conditions. Figure 3 gives typical data showing a steady rise in

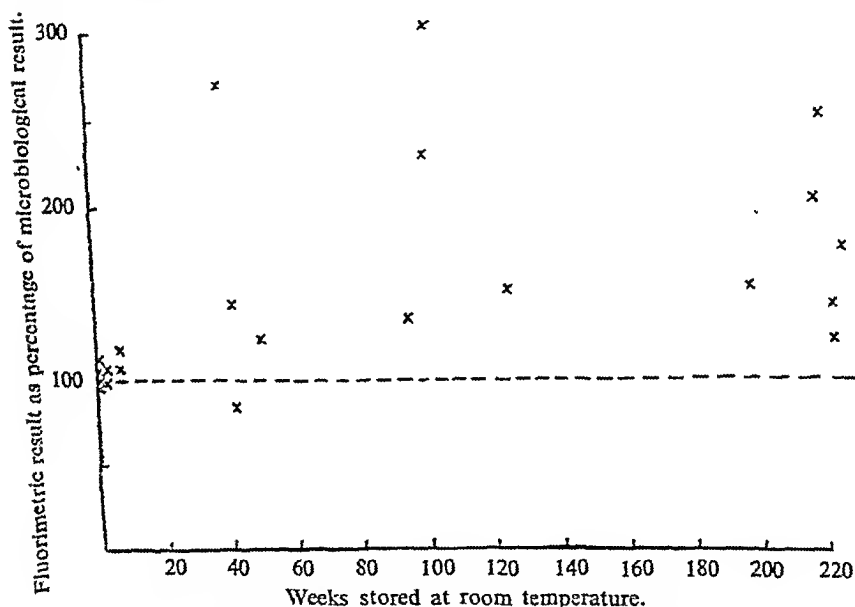


FIG. 2. Riboflavine assays on malt extract—relation between fluorimetric and microbiological results after different periods of storage.

the fluorimetric result and a steady fall in the microbiological result during $2\frac{1}{2}$ years' storage at room temperatures of a malt extract fortified with riboflavine. Similar results are being obtained with unfortified malt extract and malted preparations, but, as the riboflavine in these seems to be more stable, longer storage periods are needed for the microbiological method to show a definite loss of the vitamin.

Non-specific fluorescence in malt extract. When using the ultra-violet lamp to control our purification procedures we discovered that old malt extracts exhibit a marked blue fluorescence which is taken up by the pyridine/acetic solvent for the riboflavine, and cannot be efficiently separated from the latter by organic solvents, e.g., benzene, chloroform, ether. This blue fluorescence is much less marked in new malt extracts. It can be developed by oxidation of the malt extract (e.g., with cold

potassium permanganate), a procedure which also develops it in aqueous extracts of barley, oats and yeast. Stronger acid extracts of these exhibit, before oxidation, a non-specific yellow fluorescence which seems not to be due to riboflavin since it is only feebly absorbed by Florisil, and is converted into a blue fluorescence by permanganate oxidation. These observations indicated that the blue fluorescence is not due to lumichrome. The spectrum of the blue fluorescence has been approximately determined using the technique described by one of us (F.W.N.)⁶. Comparison of this fluorescence spectrum and that of riboflavin with the transmission curves of various secondary filters indicated that the Wratten 47 should give the greatest degree of selectivity, but could not be expected to provide completely satisfactory correction for the non-specific fluorescence. Such correction was ensured by applying the chromatographic technique described above.

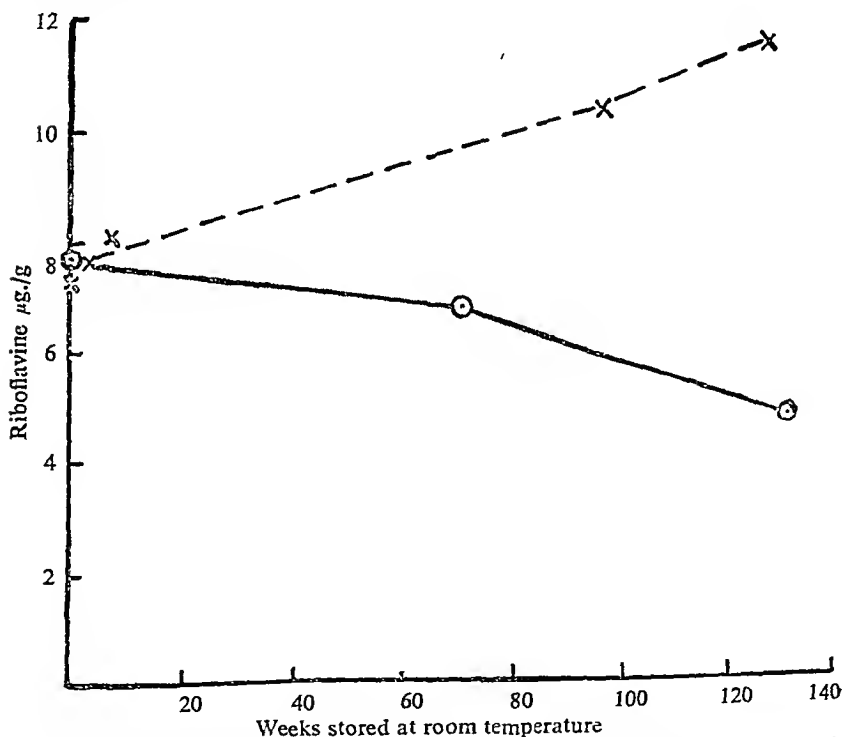


FIG. 3. Effect of storage on riboflavin content of malt extract as measured fluorimetrically X—X and microbiologically \bigcirc — \bigcirc

Effect of varying the primary filter. Our experiments with pure riboflavin solutions described in the early part of this paper showed that, as "primary filter," Wratten 47 placed behind a heat-resisting filter might be expected to give the best results. This expectation was confirmed in assays on a series of malted preparations and other foods. The average results, summarised in Table VII, showed that after adopting all the above precautions and carefully carrying out the purification

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE

procedures, the fluorimetric results on all the samples averaged 126 per cent. of the microbiological results when using Wood's glass, 107 per cent. when using Wratten 39, and 105 per cent. when using Wratten 47. The variability in results, as indicated by the standard deviation of the mean, was also lowest with the Wratten 47 filter.

Application of improved method to old malt extracts. As a final check on our improved method we applied it, using the Wratten 47 as primary filter, to a series of old malt extracts stored under known conditions for considerable periods of time. With our previous fluorimetric method these would have given results very much higher than the microbiological results. It will be seen from Table VIII that our improved method gave on this series of old malt extracts fluorimetric results in satisfactory agreement with microbiological results, and seems to have overcome the discrepancies earlier encountered by ourselves and other workers. (The table also gives details of the fluorimetric and microbiological results

TABLE VII

EFFECT OF USING DIFFERENT PRIMARY FILTERS IN FLUORIMETRIC RIBOFLAVINE ASSAYS

Sample	Fluorimetric result as percentage of microbiological result using as primary filter behind heat-resisting filter		
	Wood's glass	Wratten 39	Wratten 47
Malt and soya food	98	97	103
Malt and soya food	174	129	122
Dried yeast	115	102	92
Flour, fortified	124	101	108
Malt yeast preparation	103	90	94
Malt food A	111	102	99
" " B	103	98	100
" " C	114	109	106
Malt extract	198	138	121
Means	126	107	105
Standard deviation of mean	10.5	5.2	3.6

TABLE VIII

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS AND OTHER FOODS

Sample	Riboflavine $\mu\text{g./g.}$		Fluorimetric result as percentage of microbiological
	Fluorimetric	Microbiological	
Malt extract $7\frac{1}{2}$ years old	3.93	3.90	101
" " 7 "	4.37	4.0	109
" " 7 "	3.65	3.60	102
" " 4 " (1)	2.5	2.4	104
" " 4 "	3.15	3.10	102
" " 3 "	4.3	4.0	108
" " 3 "	5.91	5.8	102
" " $\frac{1}{2}$ " (fortified)	14.2	15.1	94
Malted preparation (2)	11.8	11.5	103
" " (3)	18.5	17.1	108
" " (3)	23.0	23.1	100
" " (3)	25.0	23.8	105
Dried yeast (4)	94.1	102.0	92
Flour, fortified (4)	3.68	3.4	108

Notes.—(1) Sample prepared for the Analytical Methods Sub-Committee of the Society of Public Analysts. (2) Prepared from malted cereals and soya for clinical trials in this country. (3) Samples of baby foods. (4) Samples obtained from the Association of Vitamin Chemists, Chicago. The average coefficient of variation for a single fluorimetric assay was 4.6 in 13 consecutive assays, and for a single microbiological assay was 5.6 in 35 consecutive assays.

on some samples mentioned in the previous table). We therefore think that the fluorimetric method we have described in this paper can be relied upon to give satisfactory results on different foods, including malted preparations, with which difficulties have previously been encountered.

DISCUSSION

We have found that in devising baby foods based on malted cereals and soya the raw materials can provide the baby's requirements of aneurine and nicotinic acid, but the content of riboflavine is more critical. By far the greater part of this vitamin in such foods comes from the malted barley, and as we have previously shown, its content of riboflavine can vary widely according to the efficiency of malting. Hence the particular need for riboflavine assays on malted products. Several years' experience with microbiological assays of riboflavine has led us to believe that these can measure the riboflavine value of a food more accurately and much more conveniently than biological assays. However, in the routine control of daily batches of food products it is not always possible to wait for the results of microbiological assays, and here fluorimetric assays are valuable.

With foods of high riboflavine content (e.g., liver, milk, yeast) good agreement with microbiological results can be provided by fluorimetric assays without resorting to chromatography for removal of interfering substances¹⁶. These, however, may need treatment with a reducing agent such as sodium hydrosulphite or stannous chloride. These workers then shook their extracts vigorously with air to regenerate riboflavine, but this procedure was not favoured by subsequent workers.

With foods of lower riboflavine content, including unmalted or malted cereals, more efficient purification has been found necessary. Decolorisation with permanganate, as applied to lyochromes by Koschara¹⁷ in 1935, was employed on foods by Connor and Straub¹⁴, who also used supersorb, a brand of fuller's earth as adsorbent, and pyridine and acetic as eluant. Amongst other adsorbents tried have been lead sulphide¹⁸, superfiltrol¹⁹ and Florisil²⁰, the two latter being special brands of fuller's earth. Several workers^{4,16} have obtained satisfactory results without using adsorbents, but not on malted preparations. Difficulties in obtaining reproducible results have been reported¹⁹. We could not find any record of other workers using the ultra-violet lamp to check their purification procedures, and detecting the non-specific blue fluorescence we have found in malted preparations. The fact that this fluorescence may be developed by permanganate emphasises the need for caution in the use of this reagent. The low solubility of the blue fluorescent substance in chloroform show it not to be lumichrome. It is most effectively been recommended for purifying extracts¹⁰.

SUMMARY

1. Using a more sensitive fluorimeter than has previously been described in this country, a method is given for estimating riboflavine in malted preparations which gives much better agreement with microbiological assays than had previously been obtained.

2. These preparations when oxidised with permanganate during the purification procedure exhibit a non-specific blue fluorescence which interferes with the fluorimetric assay. In old samples of malt extract this blue fluorescence is quite marked before permanganate treatment. The behaviour of the fluorescent substance with Florisil and its low solubility in chloroform show it not to be lumichrome. It is most effectively separated from riboflavine by adsorption on Florisil and careful elution with 1 per cent. pyridine, the process being observed continuously under the ultra-violet lamp.

3. Spectroscopic studies indicated Wratten 47 as a satisfactory primary filter. Comparison of fluorimetric and microbiological results confirmed this.

4. When calculating the results of riboflavine fluorimetric assays a method based on the assumption that the *net* fluorescence is proportional to the concentration, and using solvent blanks to determine the net fluorescence, is preferable to the method more usual in this country of using calibration curves obtained by plotting *gross* fluorescence against concentration.

We are indebted to Mr. E. J. Bowen, F.R.S., for advice, to Miss Janet Horsford and Mr. R. Evans for technical assistance, to Mr. G. Slaughter for the fluorescence spectrum of riboflavine, to Messrs. Hilger and Watts, Ltd., and Messrs. Kodak, Ltd., for data on the transmission of filters.

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be regarded as a moderate diastatic value. Before the war, when Canadian high diastatic malt was available, it was quite easy to obtain such values, but now with English barley, it was not possible to do so. In the Report published by the Pharmacopœia Commission before the war a standard of 15° Lintner was recommended. Unfortunately, owing to an oversight for which he must take the major responsibility, the method given in the B.P. was equivalent to a diastatic value of 50, but even a standard of 15° Lintner was not obtainable in these days with any malt, except in rare samples. The same applied to the nitrogen figure. With English malt, unless it was possible to select samples, a figure of 4.5 per cent. was quite often not attainable.

MR. G. E. SHAW (Runcorn) asked whether the term "soluble protein" was used to denote a more or less denatured protein. Had the authors any evidence for the presence of animal protein factor in a malt extract, and, if so, did it develop during germination? The percentage of protein required in a semi-synthetic diet was very different if there was a sufficient amount of animal protein factor present. Had the authors tried paper partition chromatography for the estimation of the riboflavine?

DR. F. WOKES, in reply, said that the diastatic value of malted barley depended on the barley which was available, and Canadian barley was higher in this respect than English barley; they had had no difficulty in obtaining diastatic values and protein contents considerably higher than the minima. On the question of the solubility test for the protein, they took the American method, which was a recognised one for solid soluble protein.

He was not quite clear what was meant by the term "animal protein factor" as applied to vegetable protein. The suggestion had been made that germination might develop this so-called factor, and it was true that there did seem to be some increase in the biological value of the proteins not only of barley but of other malted cereals after they had been malted. Whether that was due to the production of an animal protein factor, or whether it was due to the destruction of inhibitors of the type of the inhibitor in soya, he did not know.

MISS C. KLATZKIN, who also replied, said that they had not done any paper partition chromatography, because they had aimed at a quantitative method. With regard to the second yellow field as well as the blue fluorescence, they had evidence in malt extract, and more particularly in yeast, of a second yellow fluorescent material which was not riboflavine.

THE CHAIRMAN asked why the authors used the B.P.C. 1923 method for diastatic value in place of the official method.

DR. F. WOKES said that they had used it in the past and had simply continued to do so. They had done more work with the B.P.C. method than with the Lintner test.

THE CHAIRMAN, in bringing the meetings to a close, said that it only remained for him to ask the members to express their thanks to those who had contributed papers to the Conference. The Science Sessions had been exceedingly well attended, and the discussions had been most valuable.

REPORT OF A SYMPOSIUM ON THE STORAGE OF DRUGS AND MEDICINES

A SYMPOSIUM Session was held on Friday, September 16, 1949, at 9.30 a.m. Dr. Norman Evers, Chairman of the Conference, presided, and the opening speakers were Dr. T. E. Wallis, Mr. L. H. Boardman and Mr. J. B. Lloyd.

DR. WALLIS said that storage must be considered in relation to the various departments of pharmaceutical practice, viz.: (1) cultivation of vegetable drugs, (2) wholesale dealing, (3) hospital practice, (4) retail trade. The same commodity will often receive different treatment in the various circumstances, but in all of them the fundamental factors affecting the stored materials are the same. Special conditions arise from the nature of the premises and facilities peculiar to the different types of organisation. To fill in the details relevant to storage in the different circumstances one must, therefore, rely for information upon the experience of those pharmacists who are familiar respectively with the collecting and drying of crude drugs, with large warehouses, with the stock-rooms of hospitals, with the fitments of retail premises, or the packaging of goods for distribution.

Environment. The features of the environment which have a definite influence upon deterioration and storage are the following:—1. Atmospheric humidity; 2. Temperature; 3. Light; 4. Oxygen of the air; 5. Living agents of destruction; 6. Odorous commodities. Of all those factors, humidity and temperature appear to be the most important, since they not only have a direct and independent influence upon storage, but they also very largely govern the development of the numerous destructive living agents which abound everywhere.

Atmospheric Humidity. Moisture in the atmosphere is generally expressed in terms of humidity. When the atmosphere is completely saturated, the humidity is 100 per cent., when half saturated 50 per cent. and so on. If the humidity is over 75 per cent. it becomes dangerous in relation to storage. Under such conditions moisture is readily absorbed by certain chemical substances such as strong sulphuric acid, absolute alcohol, calcium chloride and salts of penicillin; moisture is also absorbed by some crude drugs such as squill, gelatin, gentian and digitalis. Some of the chemical substances, such as strong sulphuric acid and calcium chloride, will continue to absorb moisture until no more is present. On the other hand, when the humidity is low, water tends to be lost by some substances, such as crystalline borax, sodium carbonate and sodium phosphate, which contain a large proportion of water of crystallisation.

Moisture in the atmosphere is to some extent dependent upon the nature of the soil upon which the premises are built. A clay soil retains much moisture, whereas sandy soils lose moisture rapidly by drainage and, being non-colloidal, they do not tend to retain much moisture in loose association with the particles of the soil as usually occurs in a clay soil. Where underground cellars are used for storing stock, these considerations assume a major importance.

Temperature. Temperature may produce effects by itself; more frequently, however, its action is associated with other features of the environment. As pointed out by Savage in 1934, absorbent cotton wool is subject to deterioration by heat alone; a raised temperature leads to a gradual loss of absorbency, due to the effect of heat in promoting a reorientation of the molecules of fatty acid present in the infinitesimal residue of cuticle left on the hairs after processing; eventually the cotton wool becomes entirely non-absorbent. For general storage, an ideal temperature is about 55° to 65°F. (10° to 14.5°C.) which, being slightly higher than the normal temperature for a great part of the year and being maintained constant day and night, reduces the humidity and minimises the risk of sudden changes of humidity. Materials so stored therefore remain dry and tendency to attack by vegetable and animal organisms is greatly reduced, because these organisms cannot exist and multiply without sufficient moisture. For certain substances which are liable at ordinary temperatures to molecular change, such as insulin, penicillin, vaccines and antibiotics generally, storage in a refrigerator is necessary. When large refrigerators are available many drugs, such as ginger and chamomile, which are specially subject to bacterial or insect attack, may be successfully stored for long periods at the low temperature provided.

Moisture and temperature together have a combined effect upon the premises and upon the materials stored in them. For example, air at 9°C. (48°F.) contained 8.7 mg. of moisture per litre, when saturated, whereas it requires twice as much moisture (viz., 17.15 mg.) to saturate one litre of air at 20°C. (68°F.). Marked changes of temperature occur with the alternation of day and night, as well as with more occasional sudden seasonal changes. If, then, there is a sudden change in temperature from 20°C. to 9°C., as when moist warm air from outside enters a cold room or house, the air becomes over-saturated and half the moisture present is thrown out in the form of water. This water is condensed upon the walls and ceilings as well as upon the contents of the room and consequently the walls stream with moisture.

Light. Exposure to sunlight will remove the colouring-matter from many drugs, especially from those which contain chlorophyll, such as leaves and herbs in general, and also from petals of flowers which contain anthocyanin pigments or coloured plastids. This loss of colour is obvious, but other changes are induced which are not visible to the eye and sensitive constituents such as the glycosides of digitalis, may be destroyed. The vitamins as a group are sensitive to light, exposure to which ultimately leads to their destruction. The active constituents of rhubarb are orange yellow in colour and gradually change under the influence of light to a pinkish tint, giving visual evidence of deterioration. Several colourless substances acquire colour by exposure to light; santonin becomes at first yellow and gradually deepens in colour till it is almost black; silver nitrate also rapidly darkens in colour and phenol gradually becomes pink.

Oxygen of the Air. Oxidation of many active principles is brought about by atmospheric oxygen, as exemplified by the resinification of

the cannabinol of Indian hemp and the gradual loss of solubility in light petroleum of the abietic acids of colophony. Linseed oil and many volatile oils, notably oils of lemon and turpentine, resinify by exposure to the air, owing to oxidation effects. Cod-liver oil contains unsaturated fatty acids which break down by the action of oxygen causing both rancidity and resinification of the oil.

Living Agents of Destruction. Moisture, temperature and oxygen together encourage the development of many living organisms which feed upon the substances stored. Moulds of various kinds are the most important vegetable organisms and animal pests include mites, silver fishes, moths, ants, small beetles and cockroaches. The most important factor in controlling all these living organisms is moisture, for without sufficient moisture protoplasm cannot retain its life and activity. A low temperature, in the neighbourhood of $0^{\circ}\text{C}.$, is useful to prevent the development of organisms from spores and eggs, but it does not usually destroy them. The most effective means of dealing with rats and mice is to make the store proof against their entrance into it; for example, avoid wooden floors, ceilings and roofs.

Odorous Commodities. Substances such as valerian, garlic and highly perfumed soaps must be separated from one another and also from other materials in such a way as to prevent the communication of odours.

General Rules for Storage. The principal items to which attention must be directed are the following:—1. Construction of the store-room or premises; 2. Protection from dust; 3. Shelving and its arrangement; 4. Packaging; 5. Inflammable substances.

Construction of the Store-room of Premises. In general, rooms should have concrete floors and rounded corners; any crevices should be filled in with cement. Wooden plank floors should be avoided chiefly because of the number of cracks and crevices where organisms could multiply. Wooden flooring is also open to penetration by rats and mice. An equable temperature should be provided, usually cool; this is one reason why a dry cellar is often a good location for a store.

Protection from Dust. Dust contains numerous spores and small living organisms, which under favourable conditions will lead to the infestation of the stores. Dust must, therefore, be excluded as far as possible so as to avoid contamination from outside, and in this connection packing-rooms should be separate from the store. Packing materials such as straw, hay, shavings and paper should not be in or near the store; bags and sacks should also be kept outside the store. Dust also collects in the crevices and upon the ledges, grooves and guards of machinery. Machines used intermittently must not be left about in a dirty condition.

Shelving and its Arrangement. Stacks of shelving should be kept away from the walls; the lowest shelf being about 1 foot from the floor and the highest 3 feet from the ceiling. This avoids contact with condensed water streaming down walls, from moisture upon the floor and from hot moist air near the ceilings. Island stacks of shelving kept away from the walls are to be preferred to shelving actually against the walls.

Packaging and Containers. Bags and sacks should be sterilised by some process such as heating to 150°F. (65°C.) for 3 or 4 hours or by thorough washing; when filled with vegetable drugs, etc., they should be kept off the floor on a staging of slats, or they can be hung from hooks. Packages must be well closed and made of materials resistant to attack by insects and other destructive animals. Paper wrappings must be closely folded and sufficiently tightly closed to exclude moths and beetles seeking places to lay their eggs. Where the store permits access of sunlight, opaque or amber-glass containers must be used when light is deleterious to the contents.

Inflammable Substances and Poisons. Inflammable substances must be kept in a separate store well away from the main buildings. Poisons must be stored so as to comply with the regulations of the relevant legal enactments.

Control of Pests and Sterilisation of Premises. If premises or goods become infected with vegetable or animal pests, means of control and sterilisation must be adopted. It is better, however, to remember and act upon the old proverb that "prevention is better than cure."

Storage, the Pharmacopœia and the Codex. The 1948 British Pharmacopœia places increased emphasis upon the storage of drugs and chemicals, for many of which it gives instructions about storage expressed in general terms. There is also the new requirement that "vegetable drugs are required to be free from insects and other animal matter and from animal excreta." A further item to be noticed is that storage over a long period results in the deterioration of many substances having a complex molecular structure, and in certain instances, such as for some of the vaccines, the period of storage is prescribed, usually in relation also to temperature. Requirements similar to those of the British Pharmacopœia will also be appended to many of the monographs of the new British Pharmaceutical Codex.

MR. L. H. BOARDMAN said that the storage conditions laid down by the British Pharmacopœia had proved satisfactory in practice. From the manufacturer's point of view, however, storage of galenicals and pharmaceuticals covered a very wide variety of subjects.

He proposed to deal with the matter under three main headings: (1) general conditions; (2) bulk containers; (3) small containers.

1. *General Conditions.* Structure of the warehouse or factory. The drug industry includes many types of operations and utilises a great variety of basic materials, and these inherent factors within the industry should have a distinct influence upon the conditions under which drugs are stored. The most important sources of outside contamination are: (a) rodents; (b) insects, flies, cockroaches, weevils, beetles, etc.; (c) general uncleanness, from storage bins, accumulation of dust, etc.

(a) Drugs such as cascara, liquorice, senega, senna, etc., are usually stored as received and the term "good housekeeping," so popular to-day, should be ever in the minds of those responsible for the storage of raw drugs. In modern buildings, concrete, brick and steel will usually prevent

rodents, but with older buildings every effort should be made to seal all the entries, through pipes, etc., with wire mesh, and basements should be well lighted and have no dead spaces between walls, floors, etc. A regular system of pest destruction by means of a virus preparation should be undertaken as it is practically impossible to prevent access of rodents at some time or other.

(b) Prevention of insect contamination is not easy, but cleanliness of the walls and floors and the use of a mixture of pyrethrum and D.D.T., will eliminate most insect life.

(c) Other sources of contamination are the moulds, yeasts and various bacteria, and here proper sanitation in manufacturing and packaging and proper control of the manufacturing plant and the use of clean equipment will do much to prevent contamination. For storing smaller items and powdered drugs, metal bins of stainless steel, galvanised iron or black metal, with well-fitting lids, are quite suitable and preferable to wood bins. Ergot may prove difficult to store for a lengthy period, also figs and prunes which are only allocated once a year. In these cases metal bins with tight-fitting lids and the application of chloroform have proved satisfactory; also suspending a bottle of chloroform in a bin will usually maintain these drugs in good condition. Air-conditioning and the use of ozonisers are also worthy of mention.

2. *Bulk Containers.* Galenicals may be stored in bulk containers of various materials, the chief of which are wood, stainless steel, nickel, aluminium, galvanised iron and stoneware and glass-lined steel. Wood is quite good for storing many galenicals, oak being very suitable. Casks up to 200 gallons capacity can be handled fairly easily, they stand a lot of knocking about, they can be fairly easily cleaned by treating with calcium bisulphite and hydrochloric acid followed by washing and steaming. Such containers are suitable for storage of certain liquid extracts and infusions, where the alcohol content is low. Once the cask is conditioned it can be kept in use for many years for the same galenical. Stainless steel and pure electrolytic nickel make excellent storage vessels particularly for tinctures where the alcohol content is high and everything possible must be done to minimise loss. Following some years' experience it is possible to say that with alcoholic tinctures containing 60 to 70 per cent. spirit, the loss of alcohol averages about 2 per cent. when stored in wood.

There are many varieties of stainless steel, and galenicals over long periods attack some of them; or at least the appearance, aroma and flavour of the galenical is materially altered. Preparations containing methyl salicylate slowly turn pink. Oxymel of squill darkens in colour and the flavour alters; similar remarks apply to other preparations containing acetic acid. Ammoniated tincture of quinine does not keep satisfactorily. A preparation containing ammonium carbonate and senega darkens considerably and the flavour alters. These remarks apply only to a particular alloy of stainless steel which was found quite suitable for other galenicals, e.g., camphorated tincture of opium, compound syrup of glycerophosphates and compound tincture of benzoin. The particular brand of steel must be tried out over a period of time. Pure electro-

lytic nickel is suitable for tincture of belladonna, tincture of digitalis, tincture of capsicum, tincture of nux vomica, tincture of opium, tincture of squill, tincture of ginger, liquid extract of ipecacuanha, tincture of orange, ammoniated tincture of quinine, cascara preparations and compound syrup of figs. Aluminium vessels are suitable for preparations containing methyl salicylate and for senna preparation. Stone tanks can make very useful storage vessels for large quantities of materials and acid-resisting asphalt can be used for caulking the seams. Such vessels are satisfactory for storing mildly acid preparations such as oxymel of squill and compound syrup of ferrous phosphate, and can be easily cleaned. Similarly, acid preparations can be kept in glass or earthenware, but care has to be exercised in handling. Probably the ideal containers are glass-lined metal tanks which can be used for almost any type of preparation except those which are strongly alkaline. They are easily cleaned, nevertheless they have to be handled with reasonable care, otherwise the lining chips and repairs are expensive. The initial cost is also heavy. For solid preparations such as ointments, creams and confections, stainless steel, galvanised iron or glazed earthenware is satisfactory.

Temperature of Storage. In practice a temperature of about 60°F. has proved satisfactory, but conditions can sometimes be varied with advantage, e.g., tincture of capsicum is best made and stored at a lower temperature, preferably between 30°F and 40°F. as this eliminates fatty matter which otherwise may deposit during cold weather.

Time of Storage. Most liquid galenicals tend to deposit over quite a long period, probably due to slow coagulation of colloidal matter. Senna preparations are prone to deposit over a period of many months even when filtered repeatedly. Compound syrup of glycerophosphates is another preparation which may deposit after filtration, this being considerably affected by temperature. At temperatures exceeding 90°F., calcium citrate may come down and induce other salts to come down with it. In general, galenicals should be stillaged for periods from 1 to 6 months in order to have them in the best condition. Even then different conditions in the shop can bring about changes and cause deposits.

Small Containers. In most cases glass is the substance of choice, but many other materials are being used, such as aluminium (for tablets), plastic materials (for tubes and other screw-type containers), waxed board with screw caps (for ointments and confections), and metal tubes of pure tin or tin-coated lead and aluminium. For reagent bottles the use of polyethylene is being recommended and used particularly in America. Polyethylene is said to be resistant to all acids and alkalis up to temperatures of 160°F. and many organic solvents at temperatures up to 125°F. There is, of course, much less risk from the point of view of breakage. Pure aluminium containers, preferably anodised, are also being recommended as suitable containers for concentrated hydrogen peroxide, stabilised by the addition of about 4 p.p.m. of sodium stannate or sodium pyrophosphate. Pure tin tubes are satisfactory for almost all ointments and such articles as ichthammol and emulsion base oint-

ments A cheaper tube is made from tin-coated lead, but as the tin coating is not always evenly applied pitting may occur and corrosion set up with subsequent damage to the contents of the tube. Aluminium tubes are not always satisfactory, particularly with certain emulsion bases of the saturated fatty alcohol type. Lengthy shelf tests under varying conditions should be carried out before finally adopting tin-coated lead tubes or aluminium.

Although it may not be difficult to find a suitable container for small packs and to add a suitable preservative it is not always easy to obtain a suitable closure and liner to prevent possible chemical effect on the liner and cap and also to prevent fungoid growth on the liner due to alternate evaporation and condensation. Moulded caps of synthetic resin are better than enamelled metal closures. The latter frequently become scratched and also rust. Metal closures are, however, cheaper than plastic, and metal is still the most widely used material for machine-made caps to fit machine-made bottles. They are usually made of tin-plate or aluminium. On the advent of the plastic cap the double shell metal cap with smooth external finish was introduced. Later the "Unishell" type was introduced with a considerable saving in weight, and the design of the cap prevented the wads from falling out and to a large extent overcame the difficulty of rusting. For pharmaceutical purposes, however, the plastic cap has much to recommend it as it is generally more elegant in appearance.

The following is a list of some of the liners available with notes on their suitability for various purposes.

(a) *Ceresine*. These wads are made from paper impregnated with linseed oil and combinations of certain gums and is frequently supplied with a combination cork backing. In general, ceresine liners are not recommended for preparations with a high water content as mould growth is likely to develop, but to prevent this a waxed composition cork backing can be used, the wax containing 0.25 per cent. of nipagin T. Ceresine liners are suitable for solvents such as alcohols, benzene or turpentine

(b) *Blackol* liners are suitable for mildly alkaline products such as milk of magnesia and emulsions and will also stand up to liquid paraffin but are not suitable for benzene or turpentine or preparations containing them. Two other different liner facings are vinglite and whitesal which stand up to stronger acids than ceresine.

(c) *Rubber*. This is recommended for strongly alkaline products such as ammonia, it is also suitable for hypochlorites and tincture of iodine and, when suitably treated, for penicillin and its solutions. Telecothene liners have also proved very successful for tincture of iodine in tests recently carried out over a period of several months.

(d) *Tin Foil* is used for liners on products containing spirits and volatile solvents which are difficult to seal with other liners. It is also useful for cosmetic creams containing water or volatile oils.

(e) *Alkathene*. These liners are obtainable in two forms, as the pure material or as a cork agglomerate, the latter being cork dust bound together with alkathene. This is probably the best form of wad as it has greater compressibility than pure alkathene and gives a better seal for most purposes. Alkathene itself stands up to all the strong acids and is insoluble in most solvents at room temperature. Alcohol and chlorinated hydrocarbons cause some embrittlement on exposure to bright sunlight under tropical conditions. Alkathene is resistant to caustic alkali and offers complete resistance to mould growth and attack by bacteria. Solid polythene wads are useful for volatile solvents such as nail varnish. Chemically alkathene is the same as polyethylene or polythene made by catalytic polymerisation of ethylene under pressure.

For many purposes ordinary cork has much to recommend it and waxed corks are suitable for emulsions in bulk. Pulpboard and waxed paper discs are also suitable for viscous pastes, tablets and most dry products. Many tablets are, however, packed nowadays for retail sale in special envelopes, particularly for export, using plastic film such as polythene or metal foil. Subaseal rubber caps are finding increasing use both for carboys and smaller containers, particularly for mildly alkaline products such as mixture of magnesium hydroxide. All articles should be shelf tested under varying conditions for periods of 3 to 6 months.

Sodium benzoate in 1 to 1.5 per cent. solution will prevent corrosion of many metals, and metal objects wrapped in paper or cloth containing 2 to 2.5 per cent. of sodium benzoate are protected; for example, steel, copper, brass, and soldered points; in the case of aluminium, pitting is prevented. Sodium benzoate incorporated into the adhesive prevents corrosion round the edges and underneath labels.

Liquid extract of cascara has been known to deposit after 6 months' storage at about 60°F. when the temperature is reduced by a few degrees. It is probably better to cool to about 40°F. and filter to obtain a liquid extract free from deposit. The B.P. note requires filtration after 48 hours; this is not sufficient time unless a particular temperature is specified. Liquid extract of ergot is also prone to deposit at low temperature; the British Pharmacopœia specifies that it should be kept at a low temperature and it is essential that the initial storage temperature should be low. The deposit does not appear to affect the ergotoxine content of the liquid extract. Tincture of capsicum frequently clouds and deposits at temperatures lower than 60°F. and should preferably be stored at as low a temperature as possible prior to sending out. Compound infusion of gentian may deposit after 3 months' storage when the temperature is slightly lowered; calcium tartrate has been found in the deposit and at other times crystals of hesperidin. Ethereal tincture of lobelia will also deposit a wax-like substance on exposure to cold. Simple linctus of the National Formulary may show in warm weather a semi-solid mass of crystals of invert sugar. A note regarding storage could, with advantage, be put in the N.F. Mixture of magnesium hydroxide B.P. has been found to take up both arsenic and lead when stored in certain types of bottle for periods up to 3 months.

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The linctus of codeine of the National Formulary tends to ferment in hot weather, and the products of fermentation are very objectionable to taste and smell.

MR. J. B. LLOYD said that he proposed to view the subject from the standpoint of Storage in the Hospital Dispensary. In spite of the fact that the pharmacist is increasingly called upon to handle highly complex substances, many of which are inherently unstable, he thought that storage was less important than 20 years ago. The manufacturer endeavoured to produce preparations which would be stable under all possible conditions. Penicillin was perhaps the most unstable substance the pharmacist had ever been called upon to handle. For quite a time after the drug became available, it was common practice to send out injections in ice chests in order to minimise the loss of activity, but to-day it presented little or no storage problem. The material at present available could be stored at room temperature almost indefinitely; aqueous buffered injection solutions of penicillin-G could be stored at room temperature for at least 10 days, while still retaining at least 80 per cent. of their original activity. The stability of adrenaline solutions had undergone a similar improvement.

The use of preservatives and stabilising agents was not always a complete answer, as in many cases their use was, for one reason or another, undesirable. Moreover, storage often involved considerations other than the simple preservation of potency and strength. Provision must be made against contamination by dust and dirt; against chemical reaction between container and contents; against attack by insects and vermin and against reaction with atmospheric gases. Decomposition or infection by biological agencies may also have to be taken into account.

The store itself should be separate from the room in which actual dispensing was done. Adequate illumination, preferably natural, was important not only for its value in minimising mistakes due to the misreading of labels, but to keep down insect pests. Steel shelving and racking was preferable to the more usual timber. It was immune from attack by vermin, and did not provide a good foothold for climbing insects. More important, perhaps, was the fact that it is made in standard sections which may be added to at will, or transferred intact to an alternative site.

Conditions of temperature and humidity are much easier to lay down than to achieve in practice, adequate ventilation would, in general, be all that was necessary. Refrigerated storage space was, of course, required for antibiotic solutions or other biological materials. Inflammable liquids presented a serious fire risk, and statutory regulations must be observed.

Temperature was perhaps the most important factor. The higher it was the greater the velocity of chemical reaction and, within well-known limits, the growth of bacteria, moulds and yeasts. The new Pharmacopœia laid down storage conditions for quite a large range of materials.

Suitable conditions for all these substances were provided in an ordinary refrigerator. At the other end of the scale, solutions of protein hydrolysates for intravenous injection appeared to keep better at more elevated temperatures.

The Pharmacopœia was becoming increasingly concerned to specify the type of container in which substances were to be stored. Containers capable of excluding air, moisture or both were now frequently demanded, while the familiar "well-closed container" continued to be specified.

Glass was, of course, the traditional container for pharmaceuticals. Of its many advantages, not the least was that of transparency. The increasing use of parenteral solutions, however, had shown that it is not quite the inert material it was at one time thought to be, and had laid emphasis on its two principal failings, namely, its tendency to give off alkali to the contents, and the possibility of break-down with the separation of spicules. Quite recently a bottle had been submitted to him containing a solution of sodium bicarbonate which had been stored for several months, and from which a considerable quantity of glass spicules were filtered off. Had the bottle contained an opaque mixture requiring to be shaken, the consequences might have been serious. This breakdown was most apparent in containers which have undergone a heat sterilisation process, and was particularly evident in solutions of sodium citrate.

Free alkali given up to solutions from the container was provided for by an official limit test. The test, however, only applied to ampoules and similar containers of 0.5 to 25 ml. capacity. In his experience most ampoules available to-day passed the test, both on the whole ampoule and the crushed glass, but very few large containers passed when crushed. There was, in fact, no official specification for containers over 25 ml., although the United States Pharmacopœia applies a test of similar sensitivity to containers of all sizes. He suggested, therefore, that the test be extended in scope to containers of all sizes. Rubber wads, which also come into contact with the solution, varied considerably in quality, and here again an official standard would be of advantage.

Metals were rapidly coming into use as materials for containers. Distilled water stored for 12 hours in a stainless steel container had been found to have taken up lead, derived from the soldered joints. He had recently given a trial to an alloy, "Iconel," containing 80 per cent. of nickel, 14 per cent. of chromium and 6 per cent. of steel. It was more resistant to acids than monel, and seemed to offer possibilities as a bulk container for use during the preparation of large batches of sterile products. So far, he had not found any traces of heavy metals in a number of solutions stored for long periods. Like stainless steel, however, it was subject to attack by the halogens.

In these days of injections it was highly important that contamination from dust and dirt should be reduced to a minimum. From an examination of filter residues, it was apparent that many substances had

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spent at least some portion of their existence in a hessian sack. For soluble substances, which were filtered during the process of preparing the injection, this did not matter a great deal, but in the case of insoluble powders it might present a serious risk. Would it not be possible for manufacturers to make a special "parenteral grade"? Nothing more than B.P. standard of purity would normally be required, but care should be taken to see that contamination by foreign matter was reduced to the lowest possible level.

In the dispensary, dust cover stoppers should be used. Ointments presented a difficult problem, made worse by the traditional stone jar with paper cover, in which they are sent out by the wholesaler. A little reflection would bring to mind many instances in which packaging has made no progress during the last 50 years, and this in spite of the new and cheap materials which have become available. What was needed was a new approach to the problem. In the case of proprietary and branded goods, the incentives towards good packaging have produced remarkable results, but with the ordinary run of drugs, which will form the major portion of the stock of the dispensary while extemporaneous dispensing continues to exist, the position is not nearly so good. Some minor suggestions which quickly spring to mind are the packing of soft extracts, storax, caramel, etc., in collapsible tubes; the use of stoppers containing silica gel or other dehydrating material, and a host of similar ideas which in the proprietary field have become commonplace.

The CHAIRMAN, in inviting discussion, pointed out that the subject of preservatives had been discussed a year ago. It was impossible, of course, to separate preservatives entirely from the question of storage. Contributions from younger members would be particularly welcome.

MR. H. M. HIRST (Scarborough) had distinct recollections of John Whitfield, his chief nearly fifty years ago, coming to the shop in the morning and going straight to the powdered capsicum bottle and shaking it up. He himself still did that, because otherwise a mould would soon grow on the surface. In recent years it had become difficult to keep linseed meal since now it was wanted only once in three months. Glycerin and rose water would not develop a fungoid growth if made with glycerin of borax instead of glycerin. Why was compound syrup of glycerophosphates specially liable to develop mould? Points he had learned as an apprentice were never to refill a half empty bottle of sal volatile or ammoniated solution of quinine, and always to keep spirit of nitrous ether and hydrocyanic acid in inverted bottles. In many shops it would be impossible to carry out the suggestions which the opening speakers had made. Pyrethrum and D.D.T. mixed with derris was the ideal pesticide. Virus had been recommended for dealing with mice, but for mice and rats there was nothing to beat an old potato scooped out with arsenic in it.

For silver fishes sodium fluoride was best. For wasps carbon disulphide and carbon tetrachloride were better than the dangerous cyanide.

MR. A. W. BULL (Nottingham) agreed with Dr. Wallis that in many cases humidity associated with temperature was a potent source of deterioration. Where moisture could get in, i.e., where air could get in, with fluctuation of temperature there was a constant interchange of atmosphere over the stored material in the container, and therefore the more uniform the temperature of the warehouse or stockroom the less would be that atmospheric exchange and the better the storage conditions. Many packs which to all external appearances were perfectly sealed did in fact permit atmospheric interchange, and that might be responsible for deterioration due to chemical changes where moisture started the action. It might also be the cause of contamination by odour if the material was stored near to strongly-smelling articles. It was essential to choose the correct cap and lining disc and to apply them in an efficient manner. Certain plastic caps, particularly those of larger diameter, tended to become loose on storage more readily than metal caps of similar dimensions. Rubber might be the source of contamination with zinc. In large-scale manufacture many of the tanks were of 1000 gal. capacity and upwards. In addition to the inside of the tank all the external fittings should be thoroughly cleansed—the measuring gauges, valves, pumps and pipe-lines. It must be possible to take them to pieces in units which were easy to handle. Valve seatings should be examined regularly and replaced at intervals. Where large quantities of penicillin lozenges were produced they should be stored in a well-sealed container in an air-conditioned room and packaged under the same conditions.

MR. R. MAXWELL SAVAGE (Barnet) said that the deterioration in surgical dressings arose from the surface chemistry of the fibre, and in particular the orientation of the fatty matter which was present. Its occurrence in any particular sample of cotton wool was almost impossible to forecast. A sample which from analysis might be expected to deteriorate quickly might remain perfectly absorbent for 7 to 10 years, while another sample which seemed to be almost identical might change in a few months. A low temperature was better for storage. This problem was more troublesome in tropical climates than in temperate climates. There was probably sufficient scientific knowledge in existence to stop the trouble altogether, and the real barrier was commercial. Once the article was properly sterilised and packed it was likely to remain sterile. These articles were apt to accumulate dust, and if that happened it was not reasonable to expect the package still to retain its sterile condition when opened by the user.

MR J. H. OAKLEY (London) said that Lithcote, a plastic material, was very economical for some purposes which did not require high precision standards; it had the disadvantage of chipping readily, but with containers which were carefully handled it gave a satisfactory lining. The polythene type of plastic was easily sprayed on, and gave a satis-

factory lining. Initially it was more expensive than Lithcote, but less liable to chip. It was soft and readily scratched. Sprayed metal—a mild steel container sprayed with tin or stainless steel—had not proved quite so successful in practice as had seemed likely. Stainless steel was useful for a variety of preparations of different pH, alkaline or acid. Polish greatly influenced the non-reactiveness of the stainless steel. Many stainless steels rusted if they were not highly polished. A chemical reaction seemed to be set up which pitted the stainless steel, not only shortening the life of the vessel but also giving impurities to the products which were being mixed in the steel container. In the storage of galenicals, it was important that the preparations should be issued in chronological order. Plastic wads were not very resilient and the caps tended to become loose or did not form an effective seal.

MR. R. W. GILLHAM (Leeds) referred to the bulk packing of ointments. Earthenware pots and paper covers were untidy and difficult to manage and his firm had recently adopted waxed card containers. These were unsuitable for some types of ointment, such as those with aqueous bases and with volatile constituents like methyl salicylate, but in general they were satisfactory for ointments with paraffin bases. Ointment of yellow mercuric oxide that had been packed in waxed card containers had been found to discolour in sunlight. The darkening was found by experiment to be due to the blue end of the spectrum. The same thing occurred even with opal glass containers and they had to go back to the old-fashioned earthenware pot. If a parchment cover was used it let the light through and the ointment became discoloured. Another curious example of the effect of light was with a batch of liquid paraffin which developed a peculiar odour and taste and a pale straw colour. Many makes of rubber stopper were unsuitable for iodine bottles. Brushes having aluminium stems were unsuitable; the aluminium very rapidly combined with the iodine. Little had been said about strong smelling drugs. Infants' and invalids' foods stored in cardboard containers should be well segregated from strong-smelling drugs. Selling goods in the correct time order was very important. This was emphasised in the training of apprentices, but qualified pharmacists were not always free from blame. Sometimes drugs were returned as unsatisfactory and credit was asked for them, but the batch number showed that they were made 6 or 7 years before. The top fermentation which sometimes occurred in syrups was very troublesome. Originally the liquid might be strong enough in sugar to prevent fermentation, but evaporation from the top layer, followed by condensation, gradually weakened the top layer and moulds developed. One remedy was to shake the bottles every day, as Mr. Hirst suggested should be done with capsicum. A not very satisfactory remedy when there were thousands of bottles.

MR. T. D. WHITTET (London) urged manufacturers of soluble sulphonamides to issue them in brown containers. The effect of light seemed to apply to a number of drugs containing amino groups, such as tablets

containing ethylenediamine, *para*-aminosalicylic acid and some of the sulphones. Storage problems in hospitals were of two types, bulk storage and storage in wards. Mention should also be made of containers issued to patients. It was desirable to try as far as possible to get a uniform type of storage container for the wards, with adequate labelling. The out-patient dispensary frequently gave out drugs which were to be kept by the patient for a month, and sometimes longer. Generally they used waxed cardboard containers for ointments, and often for tablets. Some of the waxed cartons were not suitable for hydrous ointment and other oil-in-water preparations, and also, for many tablets, such as tablets of acetyl- β -methylcholine.

For bulk storage, mobile shelving (Rollstores) was particularly useful. It was possible to fill a whole storage room with lines of these mobile shelves as long as a gangway was left. Stainless steel metal bins on casters were very useful. They could be pushed under a section of the metal shelving, and wheeled out when wanted. It was possible to get behind them for cleaning and to prevent insects and so on. Uniformity of appearance of storage containers in the pharmacy itself was of importance. Tablet containers which resembled a book about the size of the B.P., with a space for a label, made it possible to store large quantities of tablets in a small space. They had found a series of metal trays very useful for ampoules and small proprietaries. Each tray would take a couple of boxes of a certain size of ampoule and could be labelled and put in a cupboard, where they were easily accessible. With regard to the question of standards for blood bottles, a committee had been set up three years ago and had held sporadic meetings. It was hoped to standardise $\frac{1}{2}$ -litre and 1-litre blood bottles, of the same height and neck dimensions, so as to have a standard bottle for any transfusion fluid. The trouble had been the glass itself.

MISS V. W. BURRELL (Pinner) said that in a great many cases discoloration on storage was due not only to adverse conditions of temperature, oxidation and moisture, but also to traces of heavy metals such as copper and iron. Ascorbic acid, aneurine hydrochloride and ethyl oleate were instances. Sometimes the connecting bends or screws used in stainless steel vessels were made of brass and not entirely stainless, and this could easily be overlooked. In solutions for injection, where the prevention of discolouration was important. Seitz pads were responsible for traces of iron and, particularly, of calcium which caused precipitation with ethanalamine oleate. Calcium and zinc from rubber stoppers caused turbidity. Polyvinyl chloride liners would withstand steaming. Polythene did not stand temperatures above 100°C.

MR. R. L. STEPHENS (Brighton) said that plastic caps made with wood flour as a filler absorbed water in high humidity and gave it off in low humidity, causing a dimensional change which might be as great as 4 per cent. and which was quite sufficient to cause loosening. In that respect, metal caps were superior and when using the latter it was necessary to

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choose a pliable rubber wad which would allow for the change. Cork had been used but many alcoholic pharmaceutical preparations destroyed its resilience. Lithcote and other plastic materials had the advantage of cheapness, and although they did tend to chip easily they could be fairly readily repaired. Bakelite and Lithcote were thermosetting plastics. Polyethylene as thermoplastic material is also known as Alkathene. Polythene is the trade name for the Telegraph Construction and Maintenance Company's grade of polyethylene. Polythene was resistant to chemicals, but its resilience was not sufficient to counteract the movement of the cap, nor to take up the irregularities round the top of the bottle. It was useful for carboy stoppers and in the form of polythene film as a liner for metal boxes. Plasticised polyvinyl chloride loose liners could be used over and over again; they would keep out vermin, and would not contaminate the contents. Unplasticised polyvinyl chloride was a rigid material which would stand a temperature of about 80°C. Containers and tubes could be made from the rigid polyvinyl chloride. Plasticised polyvinyl chloride was available as tubing and had the advantage over rubber of being compounded from relatively simple materials, i.e., a single chemical which had been polymerised and a plasticiser which one could specify. For chemical purposes the plasticiser should be dioctyl phthalate, which had very little odour and was non-toxic. The material was resistant to tincture of iodine. Rubber hydrochloride had come on the market in the form of Pliofilm. A big advantage was that rubber hydrochloride was resistant to the passage of moisture vapour, where the other films let moisture vapour through very readily.

He had had considerable difficulty with the deterioration of the flavour and odour of liquid paraffin emulsions. Even when stored in amber containers these gave rise to an oily, rancid flavour and a strong odour. The emulsions of the B.P. and N.F. were particularly bad.

MR. J. A. MYERS (Bradford) said that most hospitals were steam heated, and steam flies came into almost every room. Dusting with pyrethrum, derris or D.D.T. was not the complete answer. Amber glass bottles were the most suitable containers for sterile solutions of sodium citrate, and he would like to ask whether there was any white glass which was resistant to sodium citrate and did not form spicules on autoclaving. Was there a cheap canister which could be recommended for ward storage of kaolin poultice? Cotton wool in the usual packets was not suitable for export. It was amazing that it should still be stored in blue paper packets, a most unsuitable container.

MR. H. S. GRAINGER (Westminster) said that Hysil flasks were best for citrate solutions. They did not last for a great many autoclavings, but they were far better than glass bottles. Recently they had had trouble with the rubber closures used for the ordinary M.R.C. blood bottle. The rubber caps were normally treated by boiling in sodium carbonate solution for a short time and then rinsing with distilled water after being treated with a chemical detergent. The amount of permanganate

required in the B.P. test for oxidisable matter increased about tenfold after the water had been autoclaved in contact with the rubber caps and with the rubber tubing used in the drip apparatus.

MR. R. H. HENRIKSEN (London) referred to recent experience with "surplus stock" cotton wool. It was in good physical condition, but had completely lost its absorbency. In similar cases of long storage he was sure that manufacturers would be very pleased to have material returned to them.

MR. S. CANNELL (Ashton-under-Lyme) said that he could confirm that water sterilised in containers closed with rubber caps no longer passed the B.P. test. Penicillin solutions which were passed through rubber tubing, as in a drip apparatus, lost potency, and if water sterilised in rubber capped bottles was used for the preparation of penicillin solutions they might deteriorate more rapidly.

MR. E. H. REID (Dagenham) asked whether Mr. Boardman had any experience of accelerated storage tests.

DR. W. MITCHELL (London) said that the remedy for silver fish was pyrethrum as a spray or aerosol. In general, pyrethrum was extremely valuable and, if it was applied as an insecticidal fog it penetrated into the crevices, but its effect was not persistent. Some drugs had a concealed infestation. Calabar beans could look quite sound and yet be empty. The ideal plant for handling and storage was glass-lined steel or enamelled cast-iron in conjunction with glass pipe-lines. It was expensive and it was necessary to be careful about the many gaskets used in the pipe-lines. More care was necessary when using stainless steel, which was the next best thing. One had to be careful that the fabricator did not solder the seams, and that the fitters, or even the maker of the plant, did not introduce zinc or lead washers or brass screws. Lithcoting was a valuable and cheap process and did not chip so badly as some people had suggested.

DR. G. E. FOSTER (Dartford) referred to labelling. It was very difficult to stick a label on a sheet tin container so that it would not come off. They had tried many glues and pastes, but had not been able to find one which was suitable.

MISS O. B. FLETCHER (London), referring to the difficulty of citrate solutions forming glass spicules, said she had found that a successful preventive was the addition of 0.05 per cent, of citric acid to the original solution.

DR. E. F. HERSANT (Dagenham), asked over what period the loss of alcohol had been 2 per cent. It was with the larger size of wide-mouth bottles that the loosening of plastic caps was most noticeable, and he thought that it was due to changes of temperature and the difference in the coefficient of expansion between the cap and the glass bottle. That was borne out by the fact that it was mostly in the tropics that these complaints arose.

SYMPOSIUM ON THE STORAGE OF DRUGS AND MEDICINES

MR. V. REED (London) said that paraffin emulsions, when stored in the shop, seemed to develop mould more rapidly than in the case of the emulsion with phenolphthalein. He would like to know whether pyrethrum lost its activity when stored in ordinary conditions, as in the shop round. Some chemists stored it in paper or cardboard cartons.

DR. J. M. ROWSON (London) said that his experience of the storage of many hundreds of drugs was that by far the best container was the ground glass stoppered jar. Museum jars containing highly susceptible vegetable drugs had retained their contents in good condition for a very long time. Next to that he would put the Bakelite capped bottle as exceedingly efficient, especially for powders. Waxed and brown paper coverings were very unsatisfactory. For the storage of crude drugs in ordinary drawers, he had had tin containers made in three sizes, with the largest size there were four tins in the drawer; the next size divided that longitudinally so as to get eight tins in, and the next, divided also transversely, got sixteen in. To prevent insect infestation in, for example, belladonna and henbane he put 1 ml. of chloroform into each container. Perspex containers were useful, particularly for museum demonstration purposes. It was a method of display that the retail pharmacist could use more.

MR. SPEAKMAN (Birmingham), in connection with kaolin poultice, said that the makers of antiphlogistine used to put it in aluminium containers, and one could get aluminium containers holding about 1 lb. quite cheaply. They had found that polyvinyl chloride protected rubber satisfactorily against oils for 6 months.

The CHAIRMAN said that not much had been said about fermentation, which, especially during the hot summer, had been most troublesome. Some yeasts would grow in very high concentrations of sugar, but the general cause was that mentioned by Mr. Gillham—condensation in the upper part of the container forming a dilute solution on the top of the liquid. Fermentation, however, might not always be caused by yeast, and they had had a case recently of its being caused by a bacterium in malt extract. The gas produced might not always be carbon dioxide. Some time ago they had a case of fermentation in a preparation of malt extract and hæmoglobin which was caused by nitrogen-producing organisms. Rubber caps were made from an extraordinary variety of ingredients, the rubber content varying from about 20 per cent. up to nearly pure rubber, and the manufacturers were very secretive about what they put in.

DR. T. E. WALLIS, replying to the discussion, said that penetration of bags by dust, due to variations of temperature, was an important point. Museum cases were usually made "dustproof" (so called). They had some in the Society's Museum, and found that when central heating was introduced they became particularly inefficient. One of the main reasons for that was, he thought, that there was a much greater alteration in temperature inside the cases, which resulted in small currents of air going through exceedingly small cracks and producing a deposit of very

fine dust over everything, which was difficult to deal with. He thought that the reference by Mr. Whittet to steel shelving and steel containers, provided a very useful hint on how some pharmacists could considerably improve their storage conditions. He would associate with that the remarks of Dr. Rowson about the storage of drugs in tins as one of the best ways of keeping insect pests away from specimens. Storage of things like starch and chamomile in open drawers in shops was quite common, and caused deterioration. Dust and moisture got in and led to a good deal of spoilage of stock. The most important precaution to take against silver fish was to keep the place absolutely clean, and if they were found, empty the place out and whitewash it. That was an old-fashioned remedy, but it was quite a good one.

MR. L. H. BOARDMAN, who also replied, said that wood floors could always be covered with acid-resisting asphalt, which made a very satisfactory surface. Humidity and temperature were the most important factors. He thought that 60 per cent. humidity was a fairly reasonable figure to work to. Ozonisers were useful to eliminate smells; they had tried them recently and found them very satisfactory. They too had found that plastic caps tended to become loose more than metal caps, and he thanked Mr. Stephens for his explanation. It was no use buying a stainless steel tank for storage purposes unless it was welded with the material of which the tank was made. If they were soldered, they were not stainless steel tanks.

Some types of rubber stoppers had proved satisfactory for tincture of iodine over 4 to 6 months. They had found Telcathene very satisfactory, and better than anything else so far. He had not had any experience of spraying metals except for repairing copper pans, and he had not been very satisfied. He always asked for stainless steel containers rough polished or smooth polished, as the case might be. He was not sure that that was the answer to the problem of keeping these in good condition and helping the storage, but it might be part of the answer. Galenicals were always sent out in chronological order, and that was very important. If old stocks were found in the pharmacy he did not think that any blame could be attached to the manufacturer or wholesaler. Polythene and cork dust made good liners, because they were more resilient than the polythenes themselves. They had found in the past that mercuric oxide from various makers discoloured, traces of metals probably catalysing the change. They had never found liquid paraffin to go wrong, but there was no doubt that the emulsion of the N.F. did go off very quickly, and in his opinion it had not nearly enough preservative in it. The B.P. emulsion contained two preservatives, and in a much bigger proportion than that of the N.F.

He recounted an experience of spontaneous chemical reaction in some tablets of ammonium chloride and sodium nitrate when packed in a large waxed container. He would like to thank Mr. Stephens for his excellent discourse on plastics, from which he had obtained quite a few hints. Dr. Mitchell spoke highly of pyrethrum. It did not last for long, but the

knock-down effect was tremendous. When mixed with D.D.T. he had found it perfectly satisfactory without the addition of derris, which was not pleasant to handle in bulk, particularly in powdered form. A material called Tinol was very suitable for getting labels to stick on tins. The loss of alcohol to which he had referred was 2 per cent. in 12 months. Pyrethrum lost activity on storage. Ground glass stoppered jars were very good containers for drugs, but rather expensive.

MR. J. B. LLOYD, also replying, said that by the use of the mobile type of metal shelving they had been able to increase their storage capacity about 6 times for the space available. New containers should be used in replacements for wards. A standard, or several standards, for glass containers for pharmaceuticals and foods was highly desirable. The use of acid salts had done a great deal to cut down the occurrence of glass spicules in citrate solutions. Water from a glass still with double distillation, passing through a piece of rubber tube not more than 1 foot long, had failed to satisfy the B.P. test for readily oxidisable matter. That seemed to be a general failing in distilled water which came into contact with rubber. Aneurine hydrochloride solutions were particularly liable to deterioration, and after long storage a brown precipitate might occur.

The CHAIRMAN commented on the very wide range of the subjects discussed. There were still quite a number of aspects which had hardly been mentioned. The question of tropical storage, for instance, in these days of export. This was very important to manufacturers. The symposium had been not the least successful of those which had been held up to date, and he would like to thank first of all the three opening speakers for their excellent introduction and then all those who had contributed to the discussion.

RESEARCH PAPERS

THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART II

THE "NINHYDRIN-REACTING" HYDROLYTIC FRAGMENT OF VITAMIN B₁₂

BY B. ELLIS, V. PETROW AND G. F. SNOOK

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WE have previously reported¹ that hydrolysis of vitamin B₁₂ with 20 per cent. hydrochloric acid at 100°C., followed by examination of the hydrolysates by unidimensional paper-strip chromatography using the technique described by Consden, Gordon and Martin², revealed the presence of one "ninhydrin-reacting" substance which could not be identified with any of the known amino-acids. Collateral studies by Brink *et al.*³ in America, although confirming the absence of amino-acids in the hydrolysates, did not substantiate the existence of the "ninhydrin-reacting" substance. We were therefore led to re-examine and extend our observations on this fragment of the B₁₂ complex and now report further data which fully supports our earlier conclusions.

Rigorous purification of crystalline samples of vitamin B₁₂ failed to alter the pattern of our results. Paper chromatograms of the hydrolysates, as before, invariably revealed one spot on treatment with ninhydrin, the intensity of which, however, depended markedly on the nature of the irrigation solvent employed. Thus, pronounced purple spots were obtained with solvents consisting of, or containing, the aliphatic acids. Faint or hardly visible spots, in contrast, resulted when *n*-butyl alcohol or phenol were employed. Brink *et al.*³, it should be added, used phenol as their irrigation solvent, a fact no doubt explaining the difference between the two sets of results.

Again, in another series of experiments the coloured moiety¹ produced by hydrolysis of vitamin B₁₂ was quantitatively extracted from the diluted hydrolysate with *n*-butyl alcohol, and the colourless cobalt-free aqueous phase examined spectroscopically. Selective absorption in the ultra-violet was observed with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (for convenience this colourless cobalt-free hydrolytic material will subsequently be referred to as "the 285 component"), similar to certain fine structure bands present in the ultra-violet absorption spectrum of vitamin B₁₂ itself. A paper chromatogram prepared from the aqueous phase, and irrigated with *iso*-butyric acid, when developed with ninhydrin, gave the typical purple spot obtained using the total hydrolysate. A second chromatogram, run parallel with the first, when examined under a low pressure mercury resonance lamp fitted with a Corning 9863 glass filter⁴, showed a clearly visible localised pale-blue fluorescent area occupying a position corresponding to the "ninhydrin spot." The ultra-violet absorption of the eluate from this fluorescent area, moreover, showed selective absorption substantially the same as that of the aqueous solution from which the chromatogram was prepared.

ANTI-PERNICIOUS ANÆMIA FACTORS. PART II

We were thus led to believe that the "ninhydrin-reacting" substance was responsible for the fluorescence and identical with "the '285' component." In later experiments, however, when *n*-butyl alcohol-acetic acid was employed as the irrigation solvent for the chromatograms, an entirely different result was obtained. The control strip showed the "ninhydrin-reacting" area as before, but this area was no longer fluorescent when examined using the Corning filter. Furthermore, an eluate prepared from it was now found to be transparent to ultra-violet light, an observation which excludes an aromatic structure for the "ninhydrin-reacting" substance and leads to its formulation as an aliphatic base. The chromatogram, however, showed at least two blue fluorescent zones separated from each other and from the "ninhydrin-reacting" area. Eluates from these zones showed absorption spectra similar to each other and to "the 285 component," which had been resolved under these experimental conditions into at least two structurally related substances⁵.

We have already reported our failure to identify the "ninhydrin-reacting" substance with any of the naturally occurring amino-acids¹ from which it differs in giving very pale spots with ninhydrin on chromatograms irrigated with phenol (*vide supra*). In addition, studies on its chromatographic behaviour led to the tentative conclusion that the compound might be slightly volatile. We therefore turned our attention to many naturally occurring products (e.g., ethanolamine in combination with cephalin⁷; 2-aminopropanol in ergometrine⁸). The ninhydrin-reacting substance proved to be different from ethanolamine, as separation of two occurred on paper chromatograms irrigated with phenol and with collidine respectively. When the behaviour of 2-aminopropanol was investigated, R_F values identical with those of the "ninhydrin-reacting" substance were obtained on chromatograms irrigated respectively with *iso*-butyric acid, *n*-butanol-acetic acid and phenol (see experimental part). Slightly different results were given using collidine. Separation of a mixture of the two substances did not occur, a single, nearly circular spot being obtained with ninhydrin. 2-Aminopropanol itself, on the other hand, gave rise to an elongated zone.

Whilst these experiments were in progress we had noted, *inter alia*, that substantial quantities of ammonia were formed when vitamin B_{12} was heated with acid or alkali. Ammonium chloride was therefore present in acid hydrolysates of vitamin B_{12} . We therefore examined the effect of adding ammonium chloride to the 2-aminopropanol prior to paper chromatography in order to simulate the ionic environment of the "ninhydrin-reacting" substance. Irrigation with collidine, followed by development with ninhydrin, now gave a nearly circular spot identical in R_F value with the vitamin B_{12} "ninhydrin-reacting" substance. As 2-aminopropanol and the "ninhydrin-reacting" substance hydrolytic fragments appear to have identical partition coefficients in four different solvent systems, it seems difficult to avoid drawing the conclusion that the two substances are identical. A final decision on this point must, of course, rest on a rigid chemical comparison.

Finally, parallel hydrolyses of a sample of vitamin B₁₂ (Merck) (prepared by Merck & Co., Inc., and kindly sent to us through the courtesy of Dr. Randolph T. Major) and of our product, followed by detection of the "ninhydrin-reacting" fragment on paper chromatograms irrigated with 65 per cent. *iso*-butyric acid and with *n*-butyl alcohol-acetic acid respectively, have given identical results as shown in the accompanying photograph (Fig. 1). Our previous conclusions¹ are thus reaffirmed and the presence of a "ninhydrin-reacting" fragment in hydrolysates of vitamin B₁₂ firmly established.

EXPERIMENTAL

Whatman No. 1 filter paper was used for all chromatograms. Solvents for irrigation were saturated with water, with the exception of *iso*-butyric acid which was used as a 65 per cent. aqueous solution "Analar" Grade phenol was employed. Collidine (2:4:6-trimethyl pyridine) was purified by the method of Consden *et al.*⁹. Other solvents were purified by distillation. Chromatograms, after irrigation, were dried in air, sprayed with

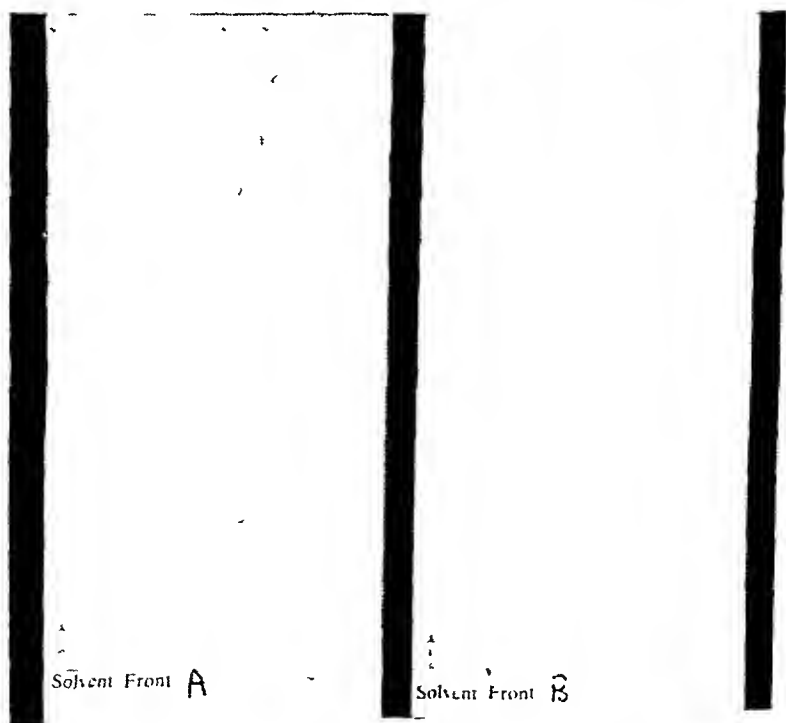


FIG. 1.—Photograph showing identity of behaviour on paper chromatograms on irrigation with (A) *isobutyric* acid and (B) *n*-butyl alcohol-acetic acid of 'ninhydrin-reacting' substances present in hydrolysates of (1) Vitamin B₁₂ (Merck) (left hand spot on each paper) and (2) Vitamin B₁₂ isolated by authors (right hand spot on each paper) Only the lower parts of the chromatograms are reproduced

a freshly prepared 0.1 per cent. solution of ninhydrin in aqueous *n*-butyl alcohol and then heated for 15 minutes at 90°C. in order to develop the colours. R_F values obtained with *iso*-butyric acid, *n*-butanol, and collidine were reproducible to within 5 per cent. Greater variations were observed with phenol, and in places of R_F values the positions of spots relative to the position occupied by valine, used as a marker, are given.

Purification of crystalline vitamin B₁₂.—A sample (30 mg.) of twice recrystallised vitamin B₁₂ was chromatographed on a column (1.4 cm. diam.) consisting of a mixture of aluminium silicate and kieselguhr. The sharply defined red band was developed to a distance of 12 cm. down the column, when it was dissected out, eluted, and the vitamin B₁₂ recovered and recrystallised twice from aqueous acetone yielding 21 mg. of repurified crystalline product.

Paper chromatography of acid hydrolysates of vitamin B₁₂.—(1) 3 mg. of the sample of crystals prepared as described above was hydrolysed for 6 hours with 0.5 ml. of 20 per cent. hydrochloric acid in a sealed tube at 100°C. The product was evaporated to dryness *in vacuo* and the coloured residue treated with 100 microlitres of distilled water. Five microlitre quantities of this solution were spotted on to paper strips in the usual way. The strips were irrigated overnight and then developed with ninhydrin with the following results.

iso-Butyric acid.—A pronounced purple spot having R_F 0.76, was obtained superimposed upon the tail of the pigment present, which formed a pale orange-red streak extending almost to the solvent front. A sample of vitamin B₁₂ (Merck), hydrolysed and chromatographed in the same way, gave an identically placed spot (paper strip A in Fig. 1).

Phenol.—The spot obtained was very weak indeed and appeared near the head of the pigment streak. It occupied a position in front of that of valine, run alongside and used as a marker. A significant change in the position of the spot did not occur when the vessel at the bottom of the chamber contained 50 per cent. acetic acid, but the intensity of the colour produced with ninhydrin was greatly increased. A weak spot was also obtained by substituting the acetic acid in the vessel by 5 N hydrochloric acid. In this case, however the "ninhydrin-reacting" substance travelled more slowly and was located a short distance behind valine, again used as a marker.

n-Butyl alcohol.—A pale spot appeared with R_F 0.20. The intensity and position was not altered by the presence of ammonia or potassium cyanide in the chamber. The pigment did not migrate.

Collidine.—A blue spot was obtained having R_F 0.34.

n-Butyl alcohol-acetic acid.—*n*-Butyl alcohol (4 vols.), acetic acid (1 vol.), and water (5 vols.) were mixed and the upper layer used as the solvent. Intense purple spots were obtained both with our sample of vitamin B₁₂ and with vitamin B₁₂ (Merck) as shown in Fig. 1 (paper strip B).

Caproic Acid.—In the one experiment carried out, a pronounced bluish-purple spot was obtained having R 0.53.

(2) Specimens of vitamin B₁₂ were hydrolysed with 20 per cent. hydrochloric acid for (a) 5 days at room temperature and (b) 6 hours at 150°C. The "ninhydrin-reacting" substance was detected on chromatograms prepared from both these hydrolysates.

(3) 3.2 mg. of vitamin B₁₂ were hydrolysed with 1 ml. of 20 per cent. hydrochloric acid by heating in a sealed tube at 100°C. for 6½ hours. The hydrolysate was diluted to 10 ml. with distilled water and a 7 ml. portion extracted 4 times with aqueous *n*-butyl alcohol (3 ml. each extraction). This treatment removed the coloured moiety completely, leaving a colourless aqueous phase the ultra-violet absorption spectrum of which showed bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å. Part of the solution was examined for the presence of cobalt with negative results. A portion (0.75 ml.) was taken to dryness *in vacuo*, the residue dissolved in 20 microlitres of water, and equal volumes (10 microlitres each) of this solution dispensed as two spots, 5 cm. apart, on a paper strip. After irrigation overnight with iso-butyric acid, the chromatogram was cut longitudinally down the centre and one half developed with ninhydrin. A purple spot appeared having R_F 0.76. Examination of the other strip under the ultra-violet light transmitted by a low-pressure mercury resonance lamp fitted with a Corning 9863 filter revealed a pale blue fluorescent spot with the same R_F value of 0.76. Black patches were not observed, thus indicating the absence of certain purines and pyrimidines.⁴ The fluorescent area of the paper was cut out, eluted with 0.1 N hydrochloric acid and the ultra-violet absorption spectrum of the eluate examined. This was substantially the same as that of the aqueous phase from which the chromatogram was prepared.

Different results were obtained by the use of *n*-butyl alcohol-acetic acid as the irrigation solvent. In this case, development of one paper strip with ninhydrin gave rise to an intense purple spot, as already described, whilst two and possibly three fluorescent spots were revealed on the companion strip when viewed under the Corning filter. These fluorescent spots were well separated from each other and also from that area of the paper containing the substance capable of giving a colour with ninhydrin. An eluate from the latter area did not show any selective ultra-violet absorption. That a "ninhydrin-reacting" substance was, in fact, present was shown by the appearance of a typical ninhydrin spot on a chromatogram prepared from this eluate.

(4) When total hydrolysates of vitamin B₁₂ were chromatographed using phenol as the irrigation solvent, the very weak spot produced with ninhydrin occupied a position in which it could easily be confused with, and even mistakenly regarded as part of, the pigment streak also present. The spot was more readily seen if the coloured moiety in a vitamin B₁₂ hydrolysate was first removed by extraction with *n*-butyl alcohol.

Comparative behaviour of the "ninhydrin-reacting" fragment and 2-aminopropanol on paper chromatograms.—5.6 mg. of vitamin B₁₂ were

hydrolysed with 20 per cent. hydrochloric acid for 6 hours in the usual way, the hydrolysate diluted, and the pigment quantitatively extracted with *n*-butyl alcohol. The product obtained on evaporation of the aqueous layer was dissolved in 250 microlitres of distilled water and this solution [solution (a)] used in the preparation of chromatograms. A 0.2 per cent. aqueous solution of 2-aminopropanol [solution (b)] was also employed.

Three spots consisting of (i) 3 microlitres of solution (a), (ii) 5 microlitres of solution (b), and (iii) a mixture of 3 microlitres of solution (a) and 5 microlitres of solution (b) were placed 2.5 cm. apart along the starting line of each paper strip (10 cm. wide and 50 cm. long). Irrigation of the strips was allowed to proceed until the solvent front had travelled in every case a distance of not less than 40 cm. The following results were obtained:

iso-Butyric acid.—The R_F values of the purple stops corresponding to (i) and (ii) were identical, namely, 0.76. A single spot corresponding to (iii) was obtained with the same R_F value of 0.76.

n-Butyl alcohol -acetic acid.—Three purple spots corresponding to (i), (ii) and (iii) were observed with identical R_F values of 0.33.

Phenol.—Acetic acid (50 per cent. v/v) was included in the chamber in order that easily visible colours should be given with ninhydrin. Valine spotted near one edge of the paper served as a marker. The apparently identical purple spots corresponding to (i), (ii) and (iii) occupied a position just in front of that of valine.

Collidine.—Both (i) and (iii) gave rise to blue spots with almost identical R_F values of ca. 0.32. 2-aminopropanol (ii) alone gave a blue elongated zone the limits of which extended from R_F 0.26 to R_F 0.44. The addition of several micrograms of ammonium chloride to spot (ii) prior to irrigation of the paper resulted in the appearance of a single nearly circular spot having an R_F value of 0.32.

Detection of ammonia formed on hydrolysis of vitamin B₁₂.—(a) On gently warming a mixture of 1 mg. of vitamin B₁₂ and 100 microlitres of N sodium hydroxide, ammonia was evolved, detected by the appearance of a brown coloration on a piece of filter paper moistened with Nessler's reagent and held over the mixture.

(b) 10 mg. of vitamin B₁₂ were hydrolysed with 0.5 ml. of 20 per cent. hydrochloric acid at 100°C. for 6 hours, the solution diluted to 10 ml., and the pigment extracted with *n*-butyl alcohol. The aqueous layer gave a colourless crystalline residue on evaporation to dryness *in vacuo*. Part of this residue was gently heated in an ignition tube with a free flame, when a white sublimate formed on the cooler upper sections of the tube. Another part of the residue, dissolved in a few microlitres of water, was mixed with one drop of cold N sodium hydroxide, and the ammonia evolved detected as described above.

SUMMARY AND CONCLUSIONS

1. Our previous conclusion that acid hydrolysis of vitamin B₁₂ leads to the formation of a "ninhydrin-reacting" substance is confirmed.
2. The behaviour of this "ninhydrin-reacting" substance and of 2-aminopropanol on paper chromatograms has been examined.
3. The results appear to show that these two substances are identical.
4. Evidence has been obtained for the presence in acid hydrolysates of vitamin B₁₂ of material showing selective absorption with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (referred to as "the 285 component").
5. This "285 component" is resolved into at least two structurally related substances by chromatography employing *n*-butyl alcohol-acetic acid as the irrigation solvent.
6. Formation of ammonia occurs during acid or alkaline hydrolysis of vitamin B₁₂.

The authors thank Mr. R. E. Rodway for technical assistance in the isolation of crystalline vitamin B₁₂ from a concentrate kindly supplied to the Research Department by Mr. A. W. Davidson. The ultra-violet absorption data were kindly determined by Dr. E. R. Holiday (M.R.C. Spectrographic Unit The London Hospital, E.1), who also carried out the examinations with the Corning filter. The authors thank the Directors of The British Drug Houses, Ltd., for permission to publish these results.

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THE CHEMISTRY OF ANTI-PERNICIOUS ANAEMIA FACTORS

PART III. 5:6-DISUBSTITUTED BENZIMINAZOLES AS PRODUCTS OF ACID HYDROLYSIS OF VITAMIN B₁₂

By G. R. BEAVEN, E. R. HOLIDAY, E. A. JOHNSON, B. ELLIS, P. MAMALIS,
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ELLIS, Petrow and Snook have shown¹ that hydrolysis of vitamin B₁₂ with 20 per cent. (w/v) hydrochloric acid at 100°C. leads to the formation of a coloured cobalt-containing complex which may be quantitatively extracted from the aqueous phase with *n*-butyl alcohol. Examination of the aqueous phase showed that, in addition to phosphate² and a "ninhydrin-reacting" substance^{1,3}, material showing selective absorption in the ultra-violet with bands and inflections at 2850, 2768, 2690, 2585, and 2500 Å ("the 285-component") was also present³.

The presence of two main absorption band systems (2850, 2768, 2690 Å) and (2585, 2500 Å) of almost equal intensity in "the 285-component" pointed to the existence of a dicyclic chromophore of unsaturated or aromatic character. The absorption spectra of aromatic compounds of this type differ from that of "the 285-component" from which it was concluded that a heterocyclic chromophore was present in the latter material. The marked fine structure of the absorption spectrum, moreover, indicated a heterocyclic compound probably containing nitrogen. On examining the absorption spectra of a number of heterocyclic compounds containing one or more nitrogen atoms in the molecule, it soon became clear that ring systems containing two fused six-membered rings could be excluded from further consideration as the absorption invariably extended to too long a wavelength. The study of compounds containing a six-membered ring fused to a five-membered ring, on the other hand, revealed the significant fact that only benziminazole (III; R=R'=H) and indazole gave spectra resembling that of "the 285-component," benziminazole approximating the more closely of the two. The characteristic long wavelength fine structure band of benziminazole corresponding to the "285"-band of the B₁₂ material, however, was at much too short a wavelength, namely, $\lambda=2730$ Å in acid solution.

While these experiments were in progress, concurrent work briefly referred to in Part II³ revealed the complex character of "the 285-component." By employing *n*-butyl alcohol-acetic acid as the irrigation solvent for the chromatograms in place of isobutyric acid which had been used for the earlier work, resolution of "the 285-component" was achieved. The chromatograms, when examined under a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter⁴, now showed three violet fluorescent zones in place of the single fluorescent area formerly obtained and ascribed to "the 285-component." A typical chromatogram is represented in Figure 1 (left hand side).

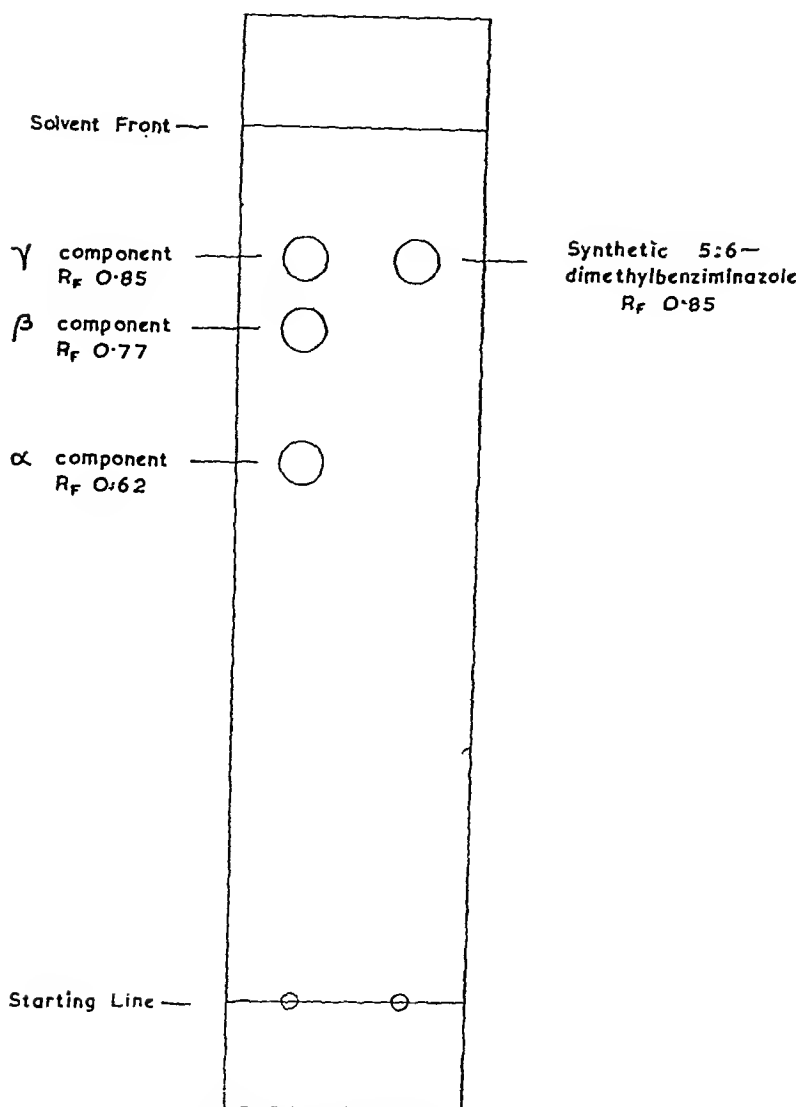


FIG. 1.—Product from vitamin B_{12} hydrolysis. Paper chromatogram irrigated with *n*-butyl alcohol-acetic acid.

Sectional elution of the different regions with dilute hydrochloric acid and spectroscopic examination of the eluates (Figure 2) showed that the compounds responsible for the three fluorescent zones had absorption characteristics similar to one another and to "the 285-component" from which they had clearly been derived. For convenience they have been designated *components* α , β , and γ . *Components* α and β , it should be added, have indistinguishable absorption characteristics which differ but slightly from those of *component* γ .

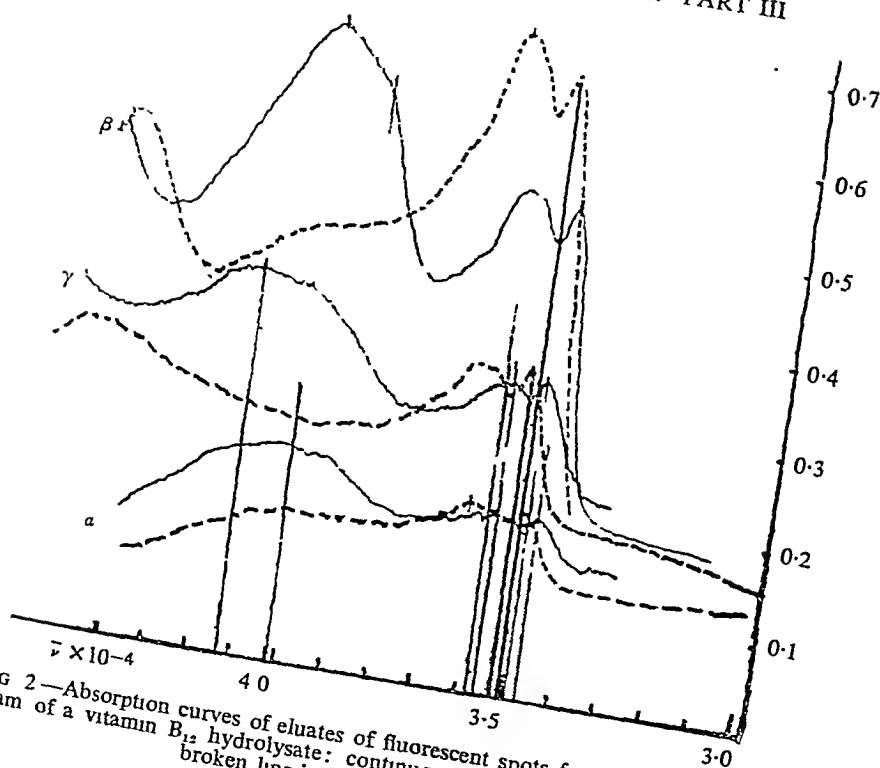
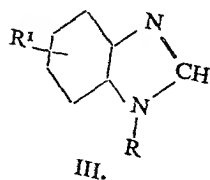
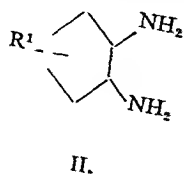
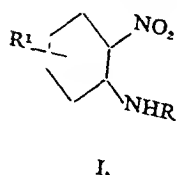


FIG 2—Absorption curves of eluates of fluorescent spots from a paper chromatogram of a vitamin B_{12} hydrolysate: continuous line in 0.01N sodium hydroxide, broken line in 0.01N hydrochloric acid.

The absorption spectra of *components* α , β , and γ bore a general resemblance to that of benziminazole (see Figure 3), but the characteristic long wavelength fine structure band of the latter compound still lay at a much shorter wavelength. The general form of the absorption spectrum of such a chromophore is usually little affected by substitution when the substituent groups are not conjugated to form a new or additional chromophore. The band systems are, however, shifted to a greater or lesser extent by substitution, usually in the direction of longer wavelengths.

These observations led us to conclude that *components* α , β , and γ were all substituted benziminazoles, and with the object of putting this theory to the test 22 alkylated benziminazoles were synthesised to serve as model compounds for spectroscopic measurements.



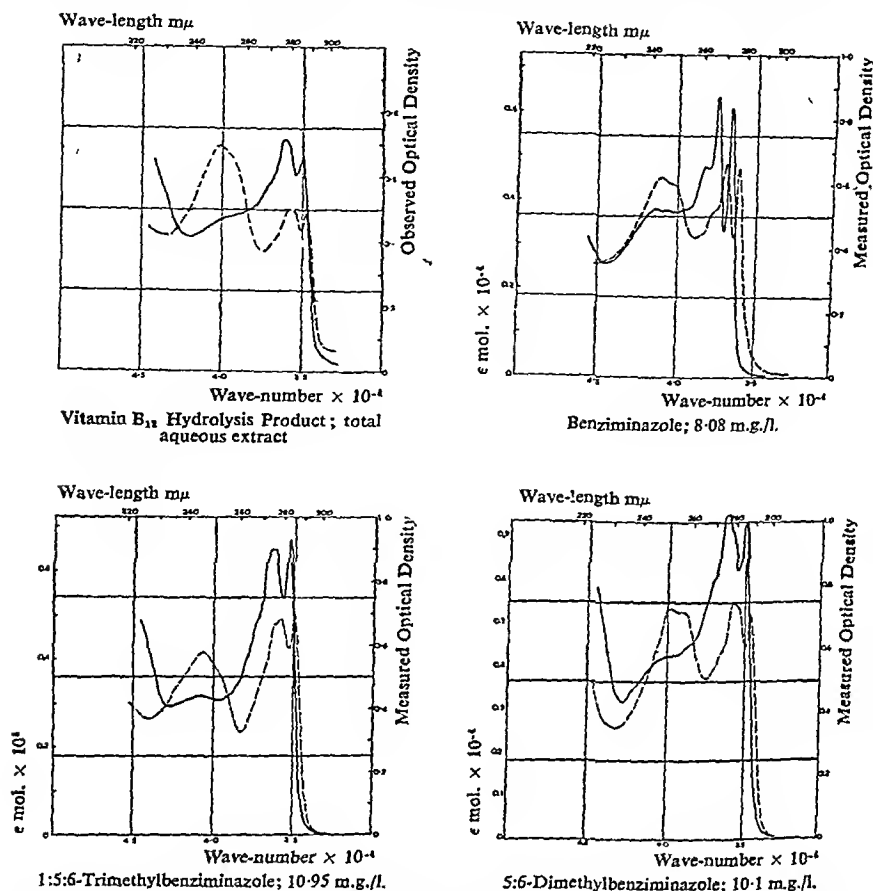


FIG. 3.—Absorption curves of benziminazoles and of the aqueous phase after *n*-butyl alcohol extraction of the vitamin B₁₂ hydrolysate: continuous line in 0.01N hydrochloric acid, broken line in 0.01N sodium hydroxide.

The general procedure of Phillips⁵ was employed for their preparation whereby the *o*-diamine (II) was heated with formic or acetic acid in the presence of 4N hydrochloric acid. The required *o*-diamines (II) were prepared by catalytic reduction of the corresponding *o*-nitroanilines (I) employing a palladium-charcoal catalyst. *N*-Methyl-*o*-nitroanilines (I; R=Me) required for the preparation of the 1-methyl substituted benziminazoles (III; R=Me) were obtained by methylation of the corresponding *o*-nitroanilines (I; R=H) using the method described by Usherwood and Whiteley⁶. The preparation and properties of the following benziminazoles have not previously been reported in the literature:

1:6-, 1:7-, 2:4-, 4:5-, 5:6-Dimethylbenziminazole.

1:2:7-, 1:4:5-, 1:5:6-, 2:4:5-, 2:5:6-Trimethylbenziminazole.

1:2:4:5-, 1:2:5:6-Tetramethylbenziminazole.

1- and 2-monomethyl and 1:2-dimethylbenziminazole, the first benziminazoles to be synthesised and examined, showed spectra resembling that of the parent ring system, but with some alteration in

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form and small shifts in wavelength which did not approach the required values of $\lambda=2850$ for components α and β , and $\lambda=2832$ for component γ (*vide infra*). A greater shift in the fine structure bands was observed with the Bz-alkylated derivatives, particularly with the 5-methyl-, 6-methyl-, 1:5-dimethyl-, and 1:6-dimethyl analogues. We therefore concentrated our synthetic efforts on the preparation of 5:6-dimethyl- and 1:5:6-trimethylbenziminazoles and, with these compounds at our disposal, were able to confirm our speculations and obtain evidence for the identity of components α and β with 1-substituted 5:6-dimethylbenziminazoles and of component γ with 5:6-dimethylbenziminazole.

The positions of the fine-structure bands observed with these two sets of compounds in both acid and alkaline solution are recorded in Table I, and the agreements between the sets of figures is indeed seen to be remarkably good.

TABLE I
POSITION OF FINE-STRUCTURE BANDS (\AA) OF HYDROLYSIS COMPONENTS OF VITAMIN B₁₂
AND OF THE CORRESPONDING METHYLATED BENZIMINAZOLES

	0.01N Hydrochloric Acid							
	a ₁	a ₂	a ₃	a ₄	a ₅	b ₁	b ₂	
α	2850	2787	2753	2683	—	—	—	
β	2851	2787	2751	2689	2657	—	—	
1:5:6 T.M.B.	2850	2787	2751	2690	2652	2597	2470	
γ	2833	2770	2731	2673	2639	2580	—	
5:6 D.M.B.	2832	2770	2730	2672	2635	2580	2440	
	0.01N Sodium Hydroxide							
	a ₁	a ₂	a ₃	a ₄	a ₅	b ₁	b ₂	b ₃
α	2880	2812	2782	—	—	—	—	—
β	2880	2816	2782	2720	2679	2558	2492	2418
1:5:6 T.M.B.	2880	2820	2784	2723	2685	2582	2498	—
γ	2861	2800	2760	2702	2660	2535	—	—
5:6 D.M.B.	2862	2802	2760	2705	2655	2535	2460	2380

T.M.B.=1:5:6-Trimethylbenziminazole

D.M.B.=5:6-Dimethylbenziminazole

Spectroscopic examination of the remaining alkylated benziminazoles provided further data supporting these conclusions. The difference between components α and β , it should be added, probably lies in the nature of the substituent groups in position 1.

All the methylated benziminazoles so far examined show characteristic spectra which are readily distinguished from each other. Each compound shows 5 or 6 bands in acid solution which are shifted in characteristic manner in alkaline solution. There are, therefore, 10 or 12 bands (12 in the case of 5:6-dimethylbenziminazole) for comparison.

As the band positions of the unknown benziminazoles agreed within the error of placing with those of the synthetic compounds, we are confident that the position of the substituents in the two sets of compounds is the same. It cannot be inferred from the identity of the spectra that the substituent groups in the vitamin B₁₂ products are methyl groups. It is certain, however, that they are small unreactive groups, otherwise distortion of the spectrum to a recognisable degree could be expected. It is hardly relevant to the present communication to report the detailed spectroscopic data for all the methylated benziminazoles synthesised and examined in the course of this investigation, but it is hoped to publish this part of the work elsewhere at a later date.

Spectroscopic identification of a compound for the structure of which there is, *a priori*, no chemical evidence, is not to be undertaken lightly. Since the method is quite empirical, one is entirely dependent on the specificity of the absorption spectra for the degree of certainty with which the positive statement of identity with a model compound may be made. The spectra are so characteristic in this series of compounds, however, that they offer more strongly presumptive evidence of identity than is usually the case. In addition, preliminary experiments on the behaviour of *component* γ and 5:6-dimethylbenziminazole on paper chromatograms support the view that the two compounds are, indeed, identical.

By using the extinction coefficient of 5:6-dimethylbenziminazole as a model for reckoning molar extinctions—an assumption justified by our observation that all the benziminazoles so far examined possess very similar molar extinction coefficients—it has been possible to determine that one molecule of vitamin B₁₂ gives rise to approximately one molecule of 5:6-dimethylbenziminazole (calc. as *components* $\alpha + \beta + \gamma$) on acid hydrolysis. This result accords with our preliminary analytical studies on the products of hydrolysis of vitamin B₁₂, and leads to the conclusion that *components* α , β and γ represent different stages of degradation of a common precursor.

Re-examination of the absorption spectrum of vitamin B₁₂ (cf. Ellis, Petrow and Snook¹) by the moving plate method⁷ shows that two bands characteristic of benziminazoles are apparent at $\lambda = 2895$ and 2785 \AA , and a trace of the third and fourth bands at $\lambda = 2630$ and 2500 \AA (see Table II). Recognition of a chromophore with a complex band system such as a benziminazole is difficult, however, as the absorption due to the rest of the molecule obscures and distorts to a great extent the absorption in the benziminazole region of the spectrum. The spectrograms reproduced in Figure 4 show, however, that the moving plate method picks out the characteristic maxima and inflections. The bands are at wavelengths considerably longer than those of *components* α , β , and γ , and, in addition, the acid-alkali shift is found to be negligible (Table II, 1 and 2). These differences might be due to the nature of the group through which the benziminazole is united to the rest of the

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molecule, or to intramolecular forces polarising the benziminazole chromophore. An example of the latter effect is to be found in the shift to longer wavelengths of the spectrum of the aromatic amino-acids in native proteins⁵, and of purines and pyrimidines in nucleic acid⁶. In both cases mild hydrolysis releases the respective fragment from the effects of these polarising forces and the spectrum reverts to that of the free chromophore.

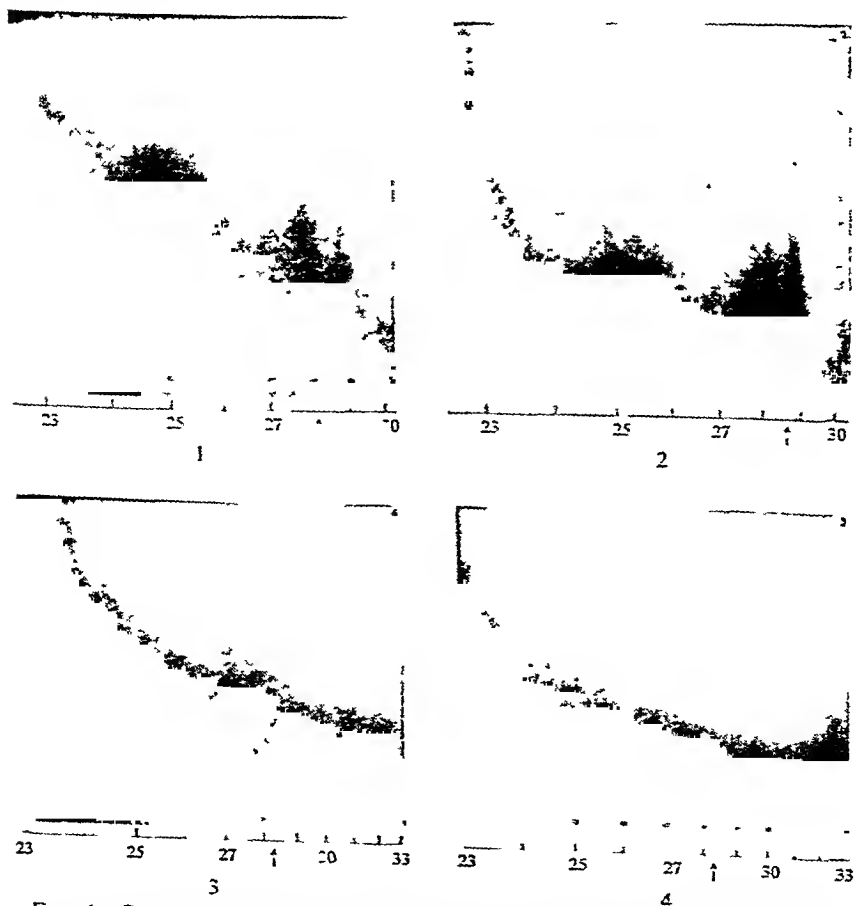


FIG. 4—Spectrophotograms of.—(1) Component β ; (2) 1:5:6 trimethylbenzimidazole; (3) Vitamin B₁₂; (4) Methyl ester of coloured component. All measurements were made in 0.01N sodium hydroxide. The arrows mark the positions of the "285-region" bands

A similar effect has now been observed in the case of vitamin B₁₂ itself. The band positions for solutions in 3N hydrochloric acid are at shorter wavelengths than those in 0.01N hydrochloric acid solutions, and do not revert to the long-wave position on returning to the latter acid concentration. The shift to shorter wavelengths observed in 3N hydrochloric acid solution is thus irreversible. Furthermore, the new

TABLE II
POSITION OF FINE-STRUCTURE BANDS (λ) OF VITAMIN B₁₂ UNDER VARYING CONDITIONS

					a_1	$a_2 + a_3$	$a_4 + a_5$	b_1	b_2
1	B ₁₂ in 0.01N sodium hydroxide	3615	3435	3235	3065	2895	2785	2630	2500
2	B ₁₂ in 0.01N hydrochloric acid	3610	3435	3235	3065	2895	2785		
3	B ₁₂ in 3N hydrochloric acid ...	3560	3415	3200	3040	2862	2770		
4	Solution (3) brought back to 0.01N hydrochloric acid ..			3210	3050	2861	2776		
5	Solution (3) brought back to weak alkali	3609	3433	3230	3070	2889	2790	2625	

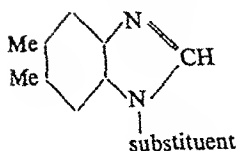
Note.—Many of the bands are difficult to distinguish. Only those which have been identified with certainty are included.

positions occupied by the bands are considerably closer to those of components α and β , and these bands now show a comparable acid-alkali shift. As a moving-plate spectrogram of a solution of the "methyl ester" of the cobalt complex also present¹ in hydrolysates of vitamin B₁₂ shows, apart from other changes, an almost complete absence of the fine structure bands in the "285-region" (see Figure 4), it may be concluded that the benzimidazole nucleus exists intact in the B₁₂ molecule.

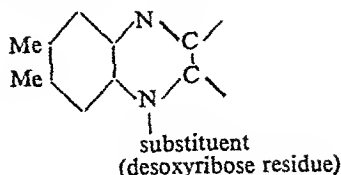
The recognition of a 5:6-dimethylbenzimidazole nucleus in vitamin B₁₂ raises a point of great biogenetic interest. Riboflavin, which likewise contains the 4:5-dimethyl-*o*-phenylenediamine residue, is known to be synthesised by microbial flora in the rumen of the sheep¹⁰. Tösis and Mitchell¹¹ have shown that the microbial flora utilise at least part of the cobalt ingested by the ruminant, and have suggested, on the basis of this observation, that pining and other wasting diseases in ruminants may be due to cobalt deficiency of the bacteria, the animal requiring not cobalt, but the bacterial products which have thereby become deficient. Becker, Smith and Loosli¹² have reported that there is no significant response in cobalt-deficient lambs when injected even with comparatively large quantities of vitamin B₁₂ (125 μ g.) or fed with B₁₂ concentrates, although rapid disappearance of the symptoms occurred following cobalt administration by feeding (1 mg. Co per day). They concluded that these preliminary observations do not support the theory that vitamin B₁₂ is an important intermediary in cobalt metabolism in lambs. Nevertheless, it seems difficult to avoid drawing the conclusion that the microbial flora of the sheep's rumen utilise cobalt to accomplish some stages in the synthesis of vitamin B₁₂ or, more probably, of a closely related derivative. What part, if any, is played by riboflavin in such a process by its action on, or part in, the bacterial metabolism will, of course, only be clarified by further experimental studies.

The formation by acid hydrolysis of vitamin B₁₂ of two 1-substituted 5:6-dimethylbenzimidazoles (components α and β) leads to the conclusion that the latter ring system is attached to the macro-fragment of the B₁₂ molecule by a grouping which is relatively stable to acid. The structural similarity between vitamin B₁₂ (IV) and riboflavin (V) implicit in

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V.

the existence of an N-substituted 4:5-dimethyl-o-phenylenediamine residue in both compounds, may well extend to the nature of this substituent. Sugar derivatives of 5:6 dimethylbenzimidazole are therefore being synthesised. Their preparation and properties will form the subject of a later communication.

EXPERIMENTAL

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Paper Chromatography of a vitamin B₁₂ hydrolysate.

1.6 mg. of vitamin B₁₂ were hydrolysed with 0.5 ml. of 20 per cent. hydrochloric acid for 15 hours at 100°C., the solution diluted to 5 ml. and extracted three times with successive portions (2 ml.) of *n*-butyl alcohol. The combined *n*-butyl alcohol extracts were back extracted several times with 2 per cent. hydrochloric acid, and all the aqueous phases combined. Evaporation of these extracts to dryness gave a crystalline residue. A solution of this material in several microlitres of distilled water was spotted on to a paper strip and the latter irrigated with *n*-butyl alcohol-acetic acid. Examination of the chromatogram in $\lambda=2536$ Å radiation revealed the presence of three violet fluorescent spots (*components* α , β , and γ) having R_F values 0.62, 0.77 and 0.85 respectively.

Chromatography of authentic 5:6-dimethylbenzimidazole gave rise to a violet fluorescent spot, identical in every respect with *component* γ (see Figure 1).

Spectrographic Methods.

(i) The moving-plate method described in 1937 by one of us⁷ has been modified by substituting a new cam which gives a ratio of final to initial rate of motion of the spectrograph plate of 100:1 instead of the original 10:1. This gives greater latitude and sensitivity to the method. The precision of location of fine-structure bands or inflections depends upon their spectral width and resolution. In the case of the sharper long wavelength bands of benzimidazoles the error is as little as ± 1 Å, while for the diffuse short wavelength bands it may increase to ± 10 Å.

(ii) The spectrophotometric measurements were made with an automatic recording spectrophotometer designed and built by the Telecommunication Research Establishment of the Ministry of Supply in collaboration with Medical Research Council. A full description of this instrument will be published elsewhere. It plots a continuous record of optical density against wave number. Figure 2 shows examples of records taken with this instrument.

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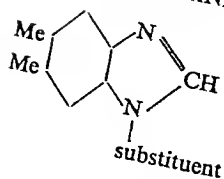
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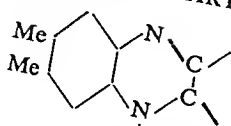
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V.
(desoxyribose residue)

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(iii) All solutions were examined in both 0.01N hydrochloric acid and sodium hydroxide. Since the change in the absorption spectrum on passing from acid to alkali is different for the different benziminazoles, the "acid" and "alkaline" spectra may be taken as independent properties of the chromophore in question and hence the specificity increased very greatly. In Figure 3 the usual notation is employed.

$$\epsilon_{\text{mol}} = \frac{1}{c \times l} \cdot D \quad \dots\dots\dots (1)$$

where ϵ is the molar extinction coefficient, D is the measured optical density, l the path length and c the concentration in g.-molecules per litre.

Samples of vitamin B₁₂ were dried at 70°C. for 1 hour prior to examination.

Spectrophotometry of Paper Chromatogram Eluates.

(i) The sensitivity of spectrophotometry:—The weight of substance (W_s , g.) to give a satisfactory optical density (D) is related to the molar extinction coefficient (ϵ_{mol}) and the volume of the photometer cell (V ml.) for a path length of 1 cm.

In most spectrophotometers the volume of solution required to fill the cell adequately is proportional to the path length. We may, therefore, define a specific volume requirement (v_s) of the photometer as the volume required to fill a cell of 1 cm. path length. Introducing W instead of c into the relation (1) we obtain

$$W_s = \frac{v_s}{1000} \frac{DM}{\epsilon_{\text{mol}}} \quad \dots\dots\dots (2)$$

where M is the molecular weight.

In our photometer 1.7 ml. is required to fill a cell of 1 cm. path length. If we take an average maximum ϵ for benziminazoles as 0.5×10^4 and a molecular weight of 150, and if we also take as a requirement that the optical density of the solution at the band maxima shall be not less than $D=0.8$, then it follows that the minimum weight of benziminazole required to fulfil the conditions is given by:

$$W_s = \frac{1.7 \times 0.8 \times 150}{1000 \times 0.5 \times 10^4} = 40 \mu\text{g.}$$

The relation (2) is general for any photometer and any compound, and is useful for rapidly determining whether the spectrophotometric method is sufficiently sensitive for estimating components of a paper chromatogram. W_s may be termed the limiting weight sensitivity of the spectrophotometer.

Where the specific absorption of a compound is high, as in the case of the benziminazoles, complete absorption curves can be derived from quantities of the order of ten micrograms.

(ii) *Identification of the components on the paper chromatogram:* Parallel runs on two spots of hydrolysate were made on the same piece

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of paper. After drying the paper in air or in the oven at 95°C., the strips were examined, before cutting the paper, under the mercury resonance arc through Corning 9863 filter which transmits the $\lambda=2536$ line (Holiday and Johnson⁴). Regions of fluorescence were observed on a typical chromatogram as described above. These were marked off with pencil, the strip cut longitudinally between the two spots, and one half sprayed with the ninhydrin reagent. The fluorescent spots were cut out from the unsprayed half, together with control sections of the paper, and each eluted by soaking at least half an hour in 4 ml. 0.01N hydrochloric acid.

It may be well to emphasise that the excitation by the $\lambda=2536$ Å radiation of the mercury arc is essential for the appearance of the fluorescent spots, which are not seen when the paper is viewed under the same arc, but through Wood's glass.

Figure 2 is a direct reproduction of the record made by the spectrophotometer on the eluates of the three fluorescent spots from such a paper chromatogram. Each pair of curves represents the same eluate in both acid and alkaline solution. In the case of each pair the spectrum shifted to longer wavelength (smaller wavenumber) is that for the alkaline solution.

Preparation of p-Toluenesulphon-o-nitroanilides:

The following general method was employed: The nitro-amine (0.1 mol.), dissolved in pyridine (15 to 20 ml.), was treated with *p*-toluenesulphonylchloride (0.1 mol.) in portions. The reaction was completed by heating for 2 hours on the steam bath. After addition of dilute hydrochloric acid, the solid was collected, washed with water and purified by crystallisation. The yields were of the order 80 to 95 per cent.

p-Toluenesulphon-(2'-nitro-4'-methylanilide), yellow needles from alcohol, m.pt. 104°C. Found: N, 9.2. $C_{14}H_{11}O_4N_2S$ requires N, 9.2 per cent.

p-Toluenesulphon-(2'-nitro-6'-methylanilide), yellow prisms from alcohol, m.pt. 125°C. Found: N, 8.8. $C_{14}H_{11}O_4N_2S$ requires N, 9.2 per cent.

p-Toluenesulphon-(2'-nitro-3':4'-dimethylanilide), prismatic needles from alcohol, m.pt. 126° to 127°C. Found: N, 8.9. $C_{15}H_{13}O_4N_2S$ requires N, 8.8 per cent.

p-Toluenesulphon-(2'-nitro-4':5'-dimethylanilide), yellow blades from alcohol, m.pt. 149° to 150°C. Found: N, 8.9. $C_{15}H_{13}O_4N_2S$ requires N, 8.8 per cent.

Methylation of the p-Toluenesulphon-o-nitroanilides: The procedure below was adopted: A mixture of the sulphonanilide (0.1 mol.) and 4N sodium hydroxide (26 ml.) was treated under reflux with methyl sulphate (8.0 ml.), the mixture being kept alkaline to phenolphthalein by drop-by-drop addition of 10 N sodium hydroxide solution. A further 8.0 ml. of methyl sulphate was added, and the mixture refluxed for 15 minutes. After cooling, the product was collected, washed with water and purified from alcohol. (Yields, 80 to 95 per cent.)

p-Toluenesulphon-(2'-nitro-N:4'-dimethylanilide), pale yellow prisms, m.pt. 128° C. Found: N, 9.1. $C_{15}H_{16}O_4N_2S$ requires N, 8.8 per cent.

p-Toluenesulphon-(2'-nitro-N:6'-dimethylanilide), silver leaflets, m.pt. 139° to 140° C. Found: N, 8.9. $C_{15}H_{16}O_4N_2S$ requires N, 8.8 per cent.

p-Toluenesulphon-(2'-nitro-N:3':4'-trimethylanilide), colourless prisms, m.pt. 137° C. Found: N, 8.7. $C_{16}H_{18}O_4N_2S$ requires N, 8.3 per cent.

p-Toluenesulphon-(2'-nitro-N:4':5'-trimethylanilide), m.pt. 125° to 127° C. Found: N, 8.3. $C_{16}H_{18}O_4N_2S$ requires N, 8.3 per cent.

Hydrolysis of the p-Toluenesulphon-N-methylanilide: A mixture of the *p*-toluenesulphon-N-methylanilide (0.1 mol.), glacial acetic acid (16 ml.) and concentrated sulphuric acid (37 ml.) was heated on the steam bath for 1 to 2 hours and poured into ice-water. The amine was collected and recrystallised from alcohol. Yields 60 to 75 per cent.

2-Nitro-N:3:4-trimethylaniline hemihydrate, scarlet prisms, m.pt. 59° to 60° C. Found: N, 14.7. $C_9H_{12}N_2O_2 \cdot \frac{1}{2}H_2O$ requires N, 14.8 per cent. Found on material resublimed at 100° C. 0.05 mm.: N, 15.5, $C_9H_{12}N_2O_2$ requires N, 15.6 per cent.

2-Nitro-N:4:5-trimethylaniline was resublimed at 100° C. 0.05 mm. for analysis, forming orange-red needles, m.pt. 138° C. Found: N, 15.6. $C_9H_{12}O_2N_2$ requires N, 15.6 per cent.

The preparation of Benziminazoles:

The appropriate nitro-amine (0.02 mol.) in ethanolic solution was shaken with hydrogen in the presence of 10 per cent. palladium-charcoal (0.5 g.) until hydrogen uptake was complete. The solution, freed from catalyst, was taken to dryness in an atmosphere of nitrogen and the residue dissolved in 4N hydrochloric acid (20 ml.). The appropriate aliphatic acid (0.1 mol.) was added and the solution refluxed in nitrogen for 40 minutes. The product was then precipitated by addition of dilute ammonia, collected and recrystallised. The yields were 50 to 60 per cent. of the theoretical.

Monomethylbenziminazoles: 1-Methyl-, needles from light petroleum, m.pt. 64° C. (Skraup¹³); 2-methyl-, needles from water, m.pt. 176° C.; 4-methyl-, needles from ethyl acetate-light petroleum, m.pt. 140° C. (Gabriel and Thieme¹⁴); 5-methyl-, needles from ethyl acetate-light petroleum, m.pt. 113° C., b.pt. 169° to 172° C./0.1 mm. (Niementowski¹⁵).

Dimethylbenziminazoles: 1:2-Dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 109° to 110° C. (Fischer¹⁶ gives m.pt. 112° C.); 1:5-dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 94° C. (Fischer¹⁷); 1:6-dimethyl-, needles from light petroleum, m.pt. 74° to 75° C. Found: C, 73.7; H, 7.0. $C_9H_{10}N_2$ requires C, 73.9, H, 6.9 per cent.) (Fischer and Wreszinski¹⁸ describe this compound as an oil, b.pt. 280° C.); 1:7-dimethyl-, prismatic needles from ethyl acetate-light petroleum, m.pt. 68° to 70.5° C. Found: N, 19.0. $C_9H_{10}N_2$ requires N, 19.1 per cent.; 2:4-dimethyl-, prisms from ethyl acetate, m.pt. 168° to 169° C. Found: C, 74.0; H, 6.6. $C_9H_{10}N_2$ requires C, 73.9; H, 6.9 per

ANTI-PERNICIOUS ANÆMIA FACTORS. PART III

cent. 2:5-dimethyl-, leaflets from ethyl acetate-light petroleum, m.pt. 202°C. (Green and Day¹⁹); 4:5-dimethyl-, leaflets from aqueous alcohol, m.pt. 196° to 197°C. Found: N, 18.8; $C_9H_{10}N_2$ requires N, 19.1 per cent.; 5:6-dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 199° to 200°C. Found: C, 73.5; H, 6.4; $C_9H_{10}N_2$ requires C, 73.9; H, 6.9 per cent.

Trimethyl benziminazoles: 1:2:5-Trimethyl-, plates from ethyl acetate, m.pt. 141°C. (Fischer and Regaud²⁰); 1:2:6-trimethyl-, rods from ethyl acetate-light petroleum, m.pt. 119° to 120°C. (Fischer and Rigaud²⁰ give m.pt. 122°C.); 1:2:7-trimethyl-, fine needles from petroleum, m.pt. 146° to 147°C. Found: C, 74.5; H, 7.7; $C_{10}H_{12}N_2$ requires C, 75.0; H, 7.6 per cent.; 1:4:5-trimethyl-, white needles from light petroleum, m.pt. 95° to 96°C. Found N, 17.7. $C_{10}H_{12}N_2$ requires N, 17.5 per cent.; 2:4:5-trimethyl-, needles from aqueous alcohol, m.pt. 188° to 190°C. Found: N, 17.1. $C_{10}H_{12}N_2$ requires N, 17.5 per cent.; 1:5:6-trimethyl-, needles from ethyl acetate-light petroleum, m.pt. 142° to 143°C. Found: N, 17.1. $C_{10}H_{12}N_2$ requires N, 17.5 per cent. 2:5:6-trimethyl-, needles from aqueous alcohol, m.pt. 233° to 234°C. Found: C, 75.2; H, 7.1; $C_{10}H_{12}N_2$ requires C, 75.0; H, 7.6 per cent.

Tetramethyl benziminazoles: 1:2:4:5-Tetramethyl-, long needles from aqueous alcohol, m.pt. 144° to 145°C. Found: N, 16.1. $C_{11}H_{14}N_2$ requires N, 16.1 per cent.; 1:2:5:6-tetramethyl-, pale yellow prisms from ethyl acetate-light petroleum, m.pt. 164°C. Found: N, 16.0; $C_{11}H_{14}N_2$ requires N, 16.1 per cent.

SUMMARY AND CONCLUSIONS

1. Evidence is presented for the presence of three chemically related substances, *components* α , β , and γ , in acid hydrolysates of vitamin B_{12} .
2. Spectroscopic examination of these components has led to their classification as benziminazole derivatives.
3. Spectroscopic comparison with 22 methylated benziminazoles synthesised to serve as model compounds, has resulted in the identification of *components* α and β as 1:5:6-trisubstituted benziminazoles, and of *component* γ as a 5:6-disubstituted benziminazole.
4. Preliminary paper chromatographic studies point to the identity of *component* γ with 5:6-dimethylbenziminazole; from which it is concluded that *components* α and β are both 1-substituted 5:6-dimethylbenziminazoles.
5. Spectroscopic and chemical evidence leads to the conclusion that only one 5:6-dimethylbenziminazole residue is released from vitamin B_{12} on acid hydrolysis and that the benziminazole nucleus exists preformed in the vitamin.
6. It is, therefore, concluded that *components* α , β , and γ represent successive stages of degradation of a common precursor.
7. The bearing of these results on the biogenesis and structure of vitamin B_{12} is briefly discussed.

p-Toluenesulphon-(2'-nitro-N:4'-dimethylanilide), pale yellow prisms, m.pt. 128° C. Found: N, 9.1. $C_{15}H_{16}O_4N_2S$ requires N, 8.8 per cent.

p-Toluenesulphon-(2'-nitro-N:6'-dimethylanilide), silver leaflets, m.pt. 139° to 140° C. Found: N, 8.9. $C_{15}H_{16}O_4N_2S$ requires N, 8.8 per cent.

p-Toluenesulphon-(2'-nitro-N:3':4'-trimethylanilide), colourless prisms, m.pt. 137° C. Found: N, 8.7. $C_{16}H_{18}O_4N_2S$ requires N, 8.3 per cent.

p-Toluenesulphon-(2'-nitro-N:4':5'-trimethylanilide), m.pt. 125° to 127° C. Found: N, 8.3. $C_{16}H_{18}O_4N_2S$ requires N, 8.3 per cent.

Hydrolysis of the p-Toluenesulphon-N-methylanilide: A mixture of the *p*-toluenesulphon-N-methylanilide (0.1 mol.), glacial acetic acid (16 ml.) and concentrated sulphuric acid (37 ml.) was heated on the steam bath for 1 to 2 hours and poured into ice-water. The amine was collected and recrystallised from alcohol. Yields 60 to 75 per cent.

2-Nitro-N:3:4-trimethylaniline hemihydrate, scarlet prisms, m.pt. 59° to 60° C. Found: N, 14.7. $C_9H_{12}N_2O_2 \cdot \frac{1}{2}H_2O$ requires N, 14.8 per cent. Found on material resublimed at 100° C. 0.05 mm.: N, 15.5, $C_9H_{12}N_2O_2$ requires N, 15.6 per cent.

2-Nitro-N:4:5-trimethylaniline was resublimed at 100° C. 0.05 mm. for analysis, forming orange-red needles, m.pt. 138° C. Found: N, 15.6. $C_9H_{12}O_2N_2$ requires N, 15.6 per cent.

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7. The bearing of these results on the biogenesis and structure of vitamin B_{12} is briefly discussed.

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

Footnote. While preparing this paper for publication we became aware at the meeting of the First International Congress of Biochemistry held at Cambridge in August, 1949, of the findings of Dr. K. Folkers and his colleagues, and at the same session we announced the conclusions we had reached in the work now reported (Holiday and Petrow, *J. Pharm. Pharmacol.*, 1949, 1, 734).

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SYNTHESIS OF THE BENZENE ANALOGUE OF VITAMIN A

By W. H. LINNELL AND C. C. SHEN

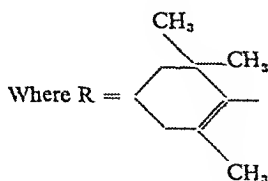
From the Pharmaceutical Chemistry Research Laboratories of the School of Pharmacy, University of London

Received October 11, 1949

THE successful synthesis of pure crystalline vitamin A by Arens and van Dorp¹ and by Isler *et al.*² opened a new field in the chemical study of vitamin A. Thus, it is possible to synthesise various analogues of vitamin A in order to establish the relationship between chemical structure and vitamin A activity. A survey of recent publications on synthetic compounds bearing modified side chains led to the conclusion that although the terminal hydroxyl group might not be of utmost importance, the length and the general skeleton of the side chain could not be altered without a complete loss of activity. (cf. Table I.)

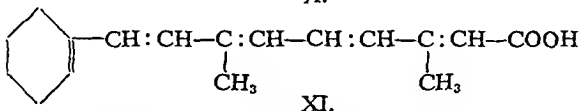
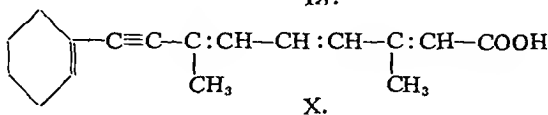
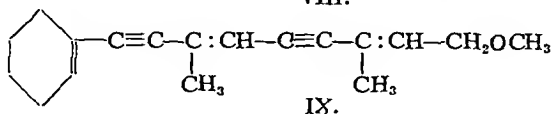
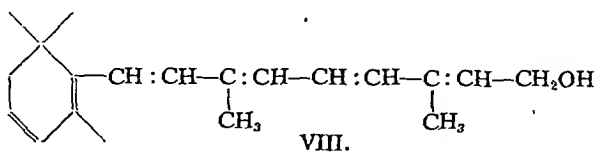
TABLE I.

Compounds		Activity (Vitamin A=1)
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{COOH} \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	I. Ref. 1	1
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}_3 \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	II. Ref. 5	1/10
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{C}-\text{C}:\text{CH}-\text{CH}_3 \\ \qquad \qquad \qquad \quad \\ \text{CH}_3 \qquad \qquad \text{CH}_3 \quad \text{CH}_3 \end{array}$	III. Ref. 5	0
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}_2\text{CH}_3 \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	IV. Ref. 5	0
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{C}:\text{CH}_2 \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	V. Ref. 5	0
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{CH}:\text{CH}-\text{CH}_2\text{OH} \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	VI. Ref. 6	1/30
$\begin{array}{c} \text{R}-\text{CH}:\text{CH}-\text{C}:\text{CH}-\text{CH}:\text{CH}-\text{CH}:\text{C}-\text{CH}_2\text{OH} \\ \qquad \qquad \qquad \\ \text{CH}_3 \qquad \qquad \qquad \text{CH}_3 \end{array}$	VII. Ref. 6	0

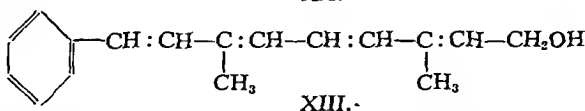
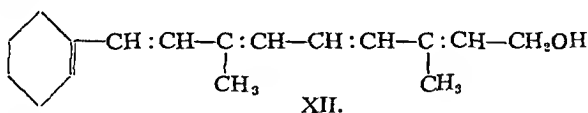


The findings of Morton *et al.*⁷ in connection with retinenes gave support to VIII as the correct structure for vitamin A₂. Nothing has yet been published on compounds having the full vitamin A side chain attached to a different nucleus. Sobotka and Chanley⁸ synthesised a cyclohexenyl analogue with two triple bonds in the side chain (IX); unfortunately, its biological activity was not published. More recently, Heilbron *et al.*⁹ reported an acyclohexenyl analogue to vitamin A acid having a single

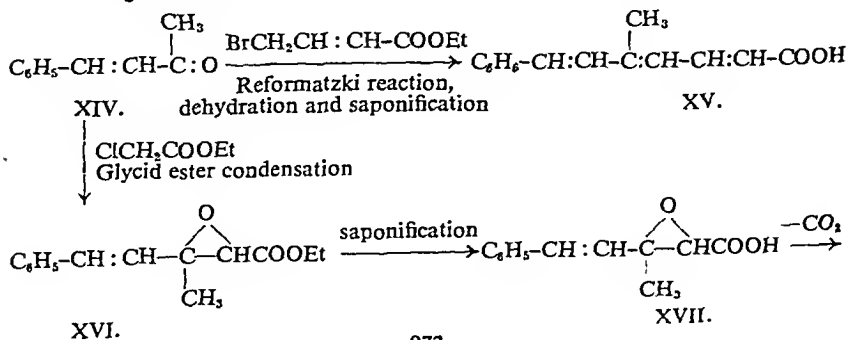
triple bond in the side chain (X) which was stated to have an activity 1/1000 that of Vitamin A. Since the presence of a triple bond next to the ring would have substantially changed the shape of the molecule, then, with the findings of Table I in mind, it may be asked whether this small activity was not due to a reduction *in vivo* into XI?



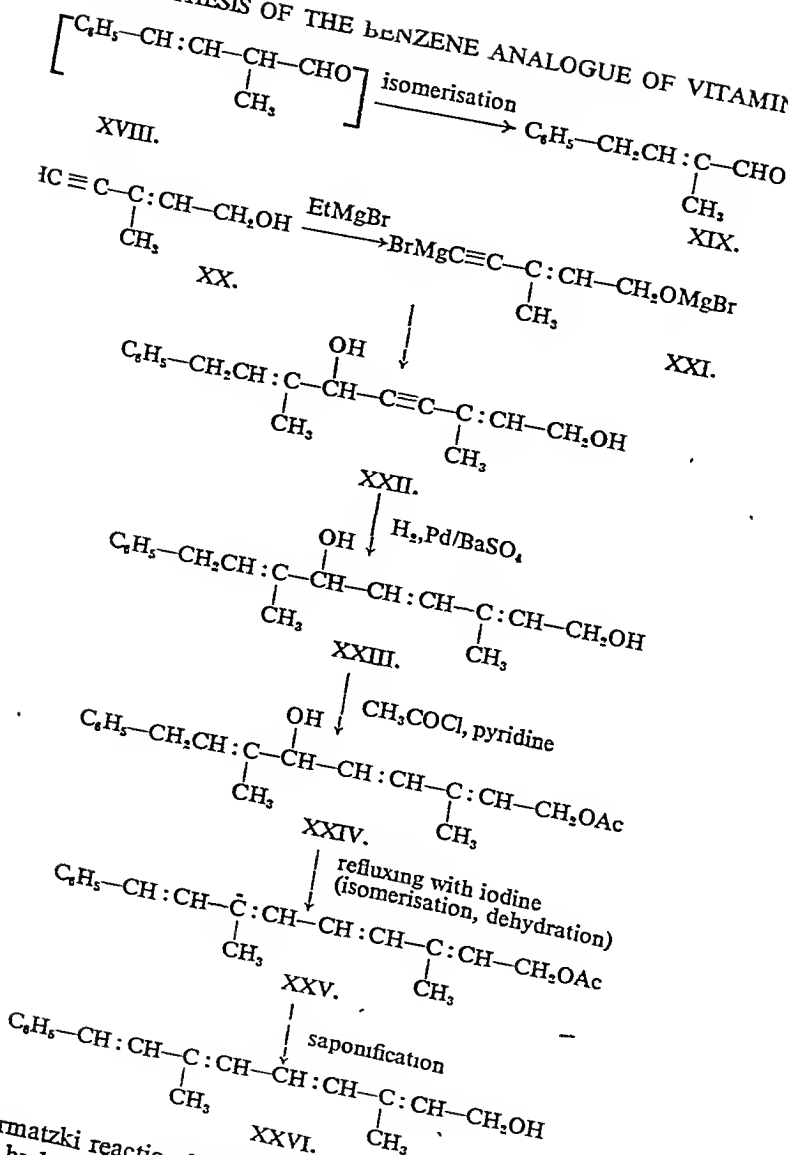
Attempts have been made in this laboratory to synthesise both the cyclohexenyl and the benzene analogue of vitamin A (XII, XIII). Owing to difficulties in preparing the key intermediate 1-Δ¹-cyclohexenyl-but-1-en-3-one, XII has not yet been obtained. However, the synthesis of the benzene analogue (XIII) has been achieved according to the route used by Isler *et al.*²



This synthesis is summarised as follows, benzalacetone being used as the starting material.



SYNTHESIS OF THE BENZENE ANALOGUE OF VITAMIN A



The Reformatzki reaction between benzalacetone and γ -bromocrotonic ester gave a hydroxy ester which was partially dehydrated upon distillation in high vacuum (10^{-4} mm. Hg pressure). After complete dehydration with anhydrous oxalic acid according to Arens and van Dorp¹, the product showed only a relatively low intensity of absorption in the ultraviolet region (max. $340\text{m}\mu$ $E_{1\%}^{1\text{cm}} = 225$) (Fig. 1, curve I). Saponification of the ester and recrystallisation of the crude gummy acid obtained from ether or acetone gave a small yield of a lemon yellow coloured crystalline acid. This acid, melting at 190° to 194°C ., gave the correct analysis for carbon, hydrogen and active hydrogen required for 5-methyl-7-phenyl-hepta-2:4:6-trienoic acid. Light absorption (Fig. 1, curve II),

in ethyl alcohol, showed a maximum at 342μ , $\log. \epsilon$ 4.548, with a subsidiary peak at 249μ . These figures are in close agreement with those expected. However, as the yield of this crystalline acid was small and subsequent work carried out with the crude acid was fruitless, a different approach to the problem was then undertaken.

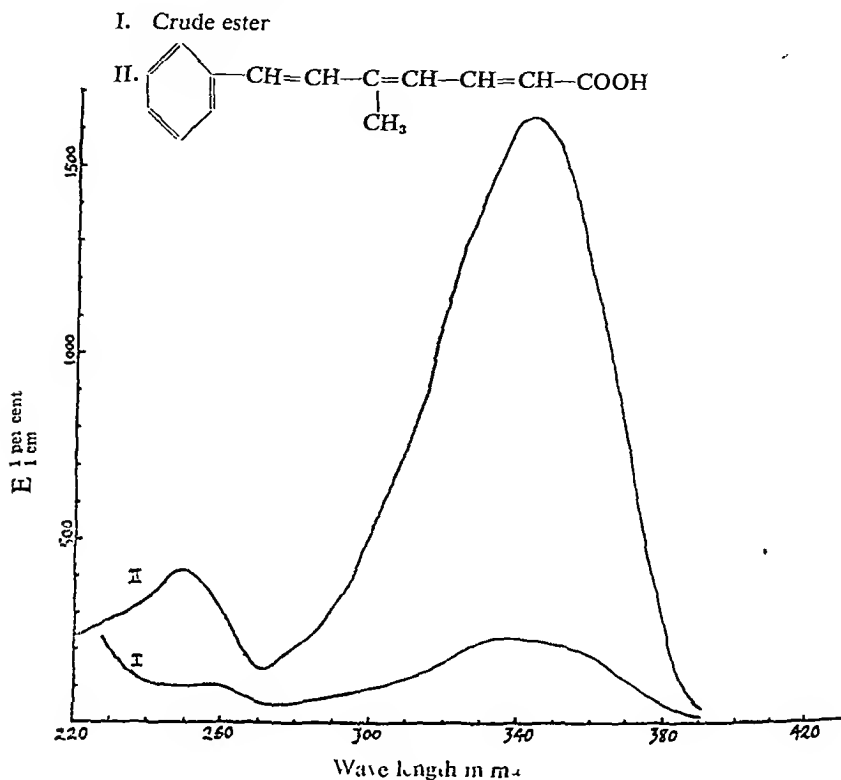


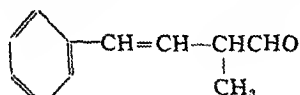
FIG. 1.

The glycid ester condensation was first carried out at -7°C . by Darzen's method¹⁰. Fractionation of the condensation product gave a 23 per cent. yield of the glycid ester (XVI) as a viscous colourless liquid boiling at 130° to 134°C . at 0.5mm. Hg pressure. Analysis gave figures in close agreement with those required. The glycid ester was found to polymerise easily on heating, redistillation at the same temperature range and under the same pressure giving only 30 per cent. recovery, the rest forming a thick oily residue in the flask, non-distillable without decomposition and solidifying into a glassy mass on cooling.

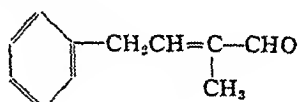
The glycid ester thus obtained was saponified by cold alcoholic potassium hydroxide, formation of some potassium carbonate precipitate indicating a partial decarboxylation during this treatment. On acidification the free glycid acid (XVII) separated as reddish precipitate and was subjected to decarboxylation without further purification. Both Heilbron *et al.*¹¹ and Milas *et al.*¹² in their preparation of the C_{14} aldehyde, isolated

the crystalline acid and decarboxylated by heating with copper or glass powder. However, with this benzene analogue, decarboxylation was found to be complete in about 15 minutes by simply heating the crude glycid acid over boiling water bath. Purification by fractionation gave about a 15 per cent. yield of the aldehyde based on the ester.

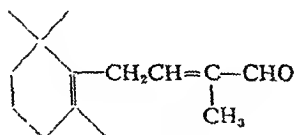
It has been observed previously that the glycid ester (XVI) was very sensitive to heat. Polymerisation during saponification and decarboxylation may be responsible for the low yield of the aldehyde. By adopting Isler's modified method⁴, the glycid ester was saponified *in situ* with alcoholic potassium hydroxide at low temperature. After working up in usual manner, a crop of pure aldehyde was obtained in an overall yield of 40 per cent. of the theoretical yield based on benzalacetone. This aldehyde, distilling at 70°C. under 0.05mm. Hg pressure showed the following characteristics: n_D^{20} 1.5537, d_4^{20} 1.0105, exaltation of molecular refraction 1.83 units. It gave analytical figures in close agreement with theory. The 2:4-dinitrophenylhydrazone occurred in red needles from chloroform, melting at 188° to 190°C. gave the correct analysis for nitrogen. The semicarbazone in leaflets from alcohol, had a melting-point 178° to 179°C. The thiosemicarbazone, needles from alcohol, had a melting-point, 132° to 132.5°C. According to classical concepts therefore, this aldehyde would be assigned formula XVIII.



XVIII.



XIX.



XXVI

But on spectroscopic examination (in ethyl alcohol), this aldehyde showed an absorption maximum at 229m μ , $\log \epsilon$ 4.214, indicating a substituted $\alpha:\beta$ -unsaturated aldehyde structure (XIX as 2-methyl-4-phenylcrotonaldehyde. A small but definite elevation at 283 to 284 m μ , $\log \epsilon$ 3.375, may correspond to the so-called R band. Its semicarbazone showed an absorption maximum at 266 m μ , $\log \epsilon$ 4.536, thiosemicarbazone at 299 m μ , $\log \epsilon$ 4.409, both typical of the corresponding derivatives of $\alpha:\beta$ -unsaturated aldehydes in general¹³. This is in agreement with Heilbron's formula for the C₁₄ aldehyde (XXVI)^{6,11} which was opposed by Milas *et al.*¹². The light absorption data for these two aldehydes and their derivatives together with those of citral are compared in Table II.

Further, if the aldehyde had the structure XVIII, it should show a maximum for the skeleton C₆H₅ - C=C - at around 290 m μ ¹⁴. Sayrene itself shows a maximum at 245 m μ , with two submaxima at 282 m μ and 290.4m μ ¹⁵. The wide differences between these figures and those obtained suggest that the aldehyde in question does not contain the

in ethyl alcohol, showed a maximum at 342μ , $\log. \epsilon$ 4.548, with a subsidiary peak at 249μ . These figures are in close agreement with those expected. However, as the yield of this crystalline acid was small and subsequent work carried out with the crude acid was fruitless, a different approach to the problem was then undertaken.

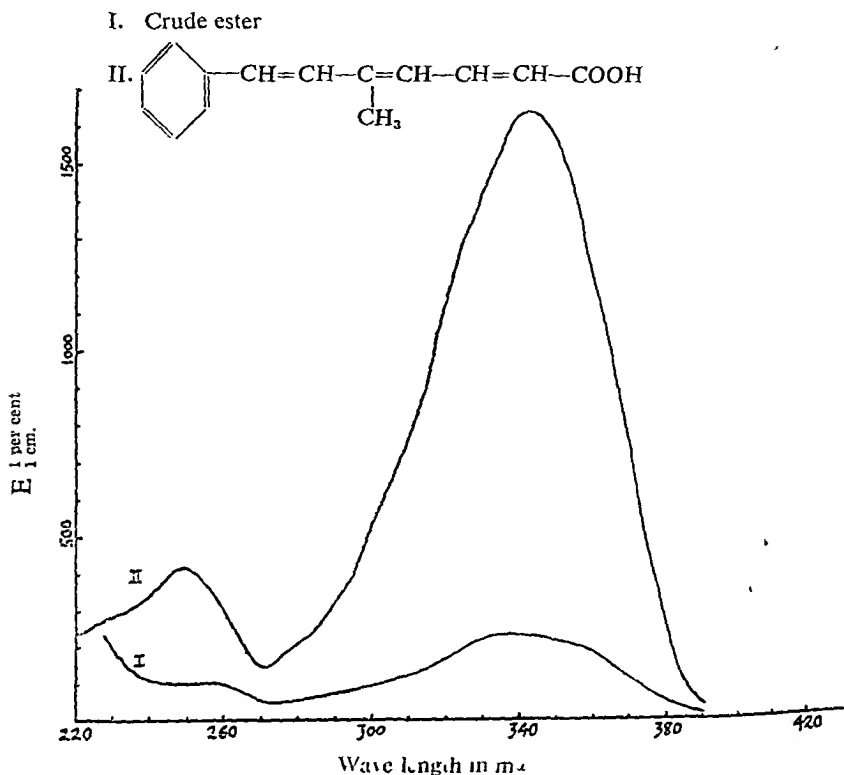


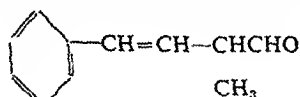
FIG. 1.

The glycid ester condensation was first carried out at -7°C . by Darzen's method¹⁰. Fractionation of the condensation product gave a 23 per cent. yield of the glycid ester (XVI) as a viscous colourless liquid boiling at 130° to 134°C . at 0.5mm. Hg pressure. Analysis gave figures in close agreement with those required. The glycid ester was found to polymerise easily on heating, redistillation at the same temperature range and under the same pressure giving only 30 per cent. recovery, the rest forming a thick oily residue in the flask, non-distillable without decomposition and solidifying into a glassy mass on cooling.

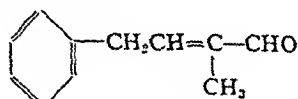
The glycid ester thus obtained was saponified by cold alcoholic potassium hydroxide, formation of some potassium carbonate precipitate indicating a partial decarboxylation during this treatment. On acidification the free glycid acid (XVII) separated as reddish precipitate and was subjected to decarboxylation without further purification. Both Heilbron *et al.*¹¹ and Milas *et al.*¹² in their preparation of the C_{14} aldehyde, isolated

the crystalline acid and decarboxylated by heating with copper or glass powder. However, with this benzene analogue, decarboxylation was found to be complete in about 15 minutes by simply heating the crude glycid acid over boiling water bath. Purification by fractionation gave about a 15 per cent. yield of the aldehyde based on the ester.

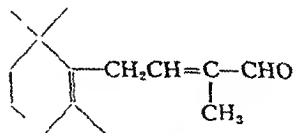
It has been observed previously that the glycid ester (XVI) was very sensitive to heat. Polymerisation during saponification and decarboxylation may be responsible for the low yield of the aldehyde. By adopting Isler's modified method⁴, the glycid ester was saponified *in situ* with alcoholic potassium hydroxide at low temperature. After working up in usual manner, a crop of pure aldehyde was obtained in an overall yield of 40 per cent. of the theoretical yield based on benzalacetone. This aldehyde, distilling at 70°C. under 0.05mm. Hg pressure showed the following characteristics: $n_D^{20^\circ\text{C.}}$ 1.5537, $d_4^{20^\circ\text{C.}}$ 1.0105, exaltation of molecular refraction 1.83 units. It gave analytical figures in close agreement with theory. The 2:4-dinitrophenylhydrazone occurred in red needles from chloroform, melting at 188° to 190°C. gave the correct analysis for nitrogen. The semicarbazone in leaflets from alcohol, had a melting-point 178° to 179°C. The thiosemicarbazone, needles from alcohol, had a melting-point, 132° to 132.5°C. According to classical concepts therefore, this aldehyde would be assigned formula XVIII.



XVIII.



XIX.



XXVI

But on spectroscopic examination (in ethyl alcohol), this aldehyde showed an absorption maximum at 229m μ , $\log \epsilon$ 4.214, indicating a substituted $\alpha:\beta$ -unsaturated aldehyde structure (XIX as 2-methyl-4-phenylcrotonaldehyde. A small but definite elevation at 283 to 284 m μ , $\log \epsilon$ 3.375, may correspond to the so-called R band. Its semicarbazone showed an absorption maximum at 266 m μ , $\log \epsilon$ 4.536, thiosemicarbazone at 299 m μ , $\log \epsilon$ 4.409, both typical of the corresponding derivatives of $\alpha:\beta$ -unsaturated aldehydes in general¹³. This is in agreement with Heilbron's formula for the C_{14} aldehyde (XXVI)^{6,11} which was opposed by Milas *et al.*¹². The light absorption data for these two aldehydes and their derivatives together with those of citral are compared in Table II.

Further, if the aldehyde had the structure XVIII, it should show a maximum for the skeleton $C_6H_5-C=CH-$ at around 290 m μ ¹⁴. Sayrene itself shows a maximum at 245 m μ , with two submaxima at 282 m μ and 290.4m μ ¹⁵. The wide differences between these figures and those obtained suggest that the aldehyde in question does not contain the

TABLE II

				$\lambda_{\text{max.}}$ m μ	log
1. 2-Methyl-4-phenyl-crotonaldehyde (XIX) (Fig. 2)	229	4.214
2. C ₁₄ aldehyde (XXVI) ⁹	230	4.25
3. Citral (commercial) ⁹	232	4.05
4. Semicarbazone of 1 (Fig. 2)	266	4.536
5. Semicarbazone of 2 ⁴	265	4.47
				269	4.462
6. Semicarbazone of 3 ⁹	272	4.498
7. Thiosemicarbazone of 1 (Fig. 2)	299	4.409
8. Thiosemicarbazone of 2 ⁹	299	4.591
9. Thiosemicarbazone of 3 ⁹	303	4.66

C₆H₅-C=C- system in its structure. This affords a more definite answer pertaining to its structure and lends indirect support to Heilbron's formula for the C₁₄ aldehyde.

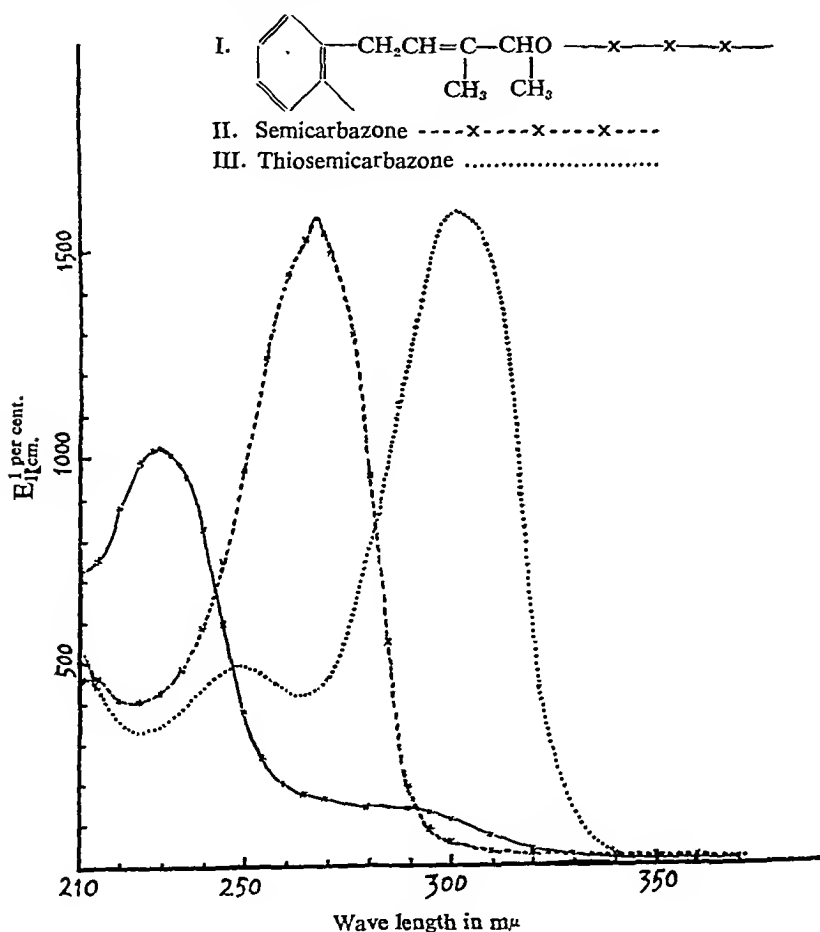


FIG. 2.

3-Methylpent-2-en-4-yn-1-ol (XX) was prepared per 3-methylpent-4-en-1-yn-3-ol by condensing methylvinyl ketone with sodium acetylide in liquid ammonia according to the method of Heilbron and Jones¹⁶. 3-Methylpent-4-en-1-yn-3-ol was found to have a refractive index $n_D^{17.5^\circ\text{C}}$ 1.4438 which is nearer to the figure given by Hennion and Leib¹⁷ ($n_D^{20^\circ\text{C}}$ 1.4444) than that by Heilbron and Jones ($n_D^{15^\circ\text{C}}$ 1.4490) although the rearranged carbinol 3-methylpent-2-en-4-yn-1-ol had $n_D^{17^\circ\text{C}}$ 1.4850, the same as that given by Heilbron and Jones.

2-Methyl-4-phenylcrotonaldehyde was coupled with the Grignard compound of 3-methylpent-2-en-4-yn-1-ol (XXI) according to Isler *et al.*⁴ In view of the fact that the reaction mixture was heterogeneous the mixture was refluxed with constant stirring for 7 hours to ensure complete reaction. After working up in usual way, the unchanged carbinol and aldehyde were removed in high vacuum. The residue so obtained was purified by partition between 75 per cent. aqueous methyl alcohol and light petroleum (40° to 60°C.) during which process the possible hydrocarbon formed was removed in the petroleum layer. The aqueous methyl alcoholic liquor was diluted with water and the oil separated was extracted with ether. Removal of the solvent gave the diol (XXII) as a viscous brownish oil in 78 per cent. yield. It showed the following characteristics: $n_D^{20^\circ\text{C}}$ 1.5756, $d_4^{19^\circ\text{C}}$ 1.0673, exaltation of molecular refraction 2.21 units. It gave analytical figures for carbon, hydrogen and active hydrogen in close agreement with theory. With antimony trichloride in chloroform it gave only a brownish black colouration. Its absorption spectrum showed inflections at 215 m μ and 230m μ which were probably due to the two isolated chromophores, the yn-en system and the benzene ring respectively. (Fig. 3, curve I.)

Semihydrogenation of the triple bond was carried out in the following way. A supported catalyst of palladium on barium sulphate (5 per cent. Pd) was prepared according to Organic Syntheses¹⁸ and was partially inactivated by the use of Rosenmund-Zetsche sulphur-quinoline poison^{19,20}. The diol (XXII) was dissolved in 10 times its volume of methyl alcohol and the hydrogenation was carried out at atmospheric pressure. This hydrogenation process was found to be extremely slow in comparison with a control experiment on 3-methylpent-2-en-4-yn-1-ol, the catalyst used being very soon completely inactivated by impurities present in the diol. Three subsequent additions of fresh partially poisoned catalyst were made and the hydrogenation stopped after nearly 13 hours when the hydrogen uptake was 0.99 mol. per mol. of the diol. The product thus obtained after the removal of catalyst and solvent gave a slightly higher carbon and lower active hydrogen figures than those required for the diol (XXIII), indicating a partial dehydration during the prolonged shaking with the catalyst. This was further confirmed by the fact that with antimony trichloride in chloroform it gave a blue colour changing rapidly into violet then red. The spectroscopic results also showed a significant change. (Fig. 3, curve II.) The maxima at 215 m μ and 240 m μ apparently due to the diol (XXIII) itself, and corresponding to the diene conjugation and the benzene ring respectively,

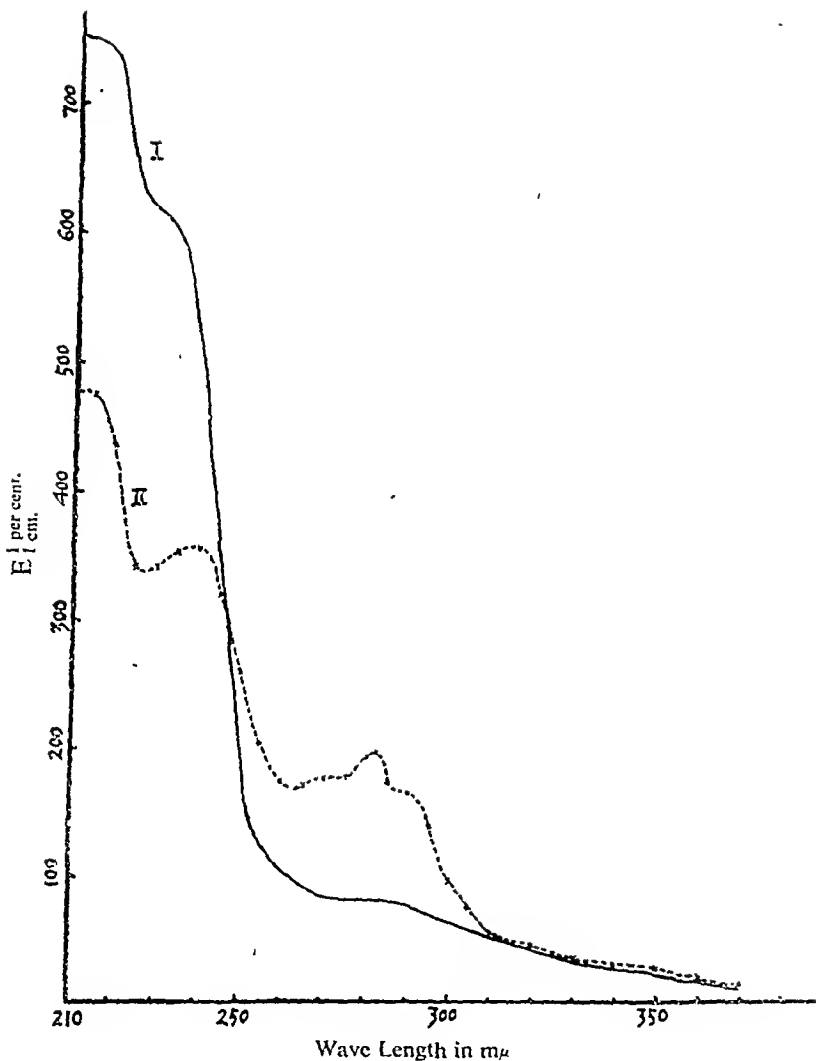
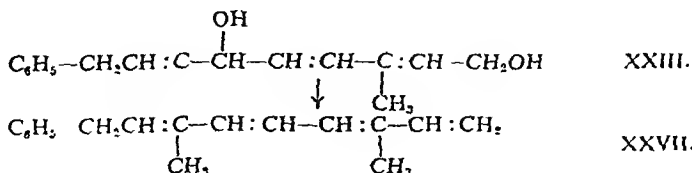


FIG. 3.

were lower than the corresponding inflections in curve I, whilst the newly formed peak at 282 $m\mu$ indicated the formation of a compound with 4 conjugated double bonds. This may possibly be explained by the formation of a hydrocarbon (XXVII) from the diol according to the following reaction:



Partial acetylation of the diol (XXIII) was carried out by treating the diol with acetyl chloride in presence of dry pyridine. The crude acetylated product gave unsatisfactory analytical figures. With antimony trichloride in chloroform it gave a transient blue colour changing rapidly into violet and red as before. Spectroscopically, it was very similar to the diol before acetylation (Fig. 4, curve I), the slight proportional increase of intensity at 282 $m\mu$ probably indicating further dehydration along the suggested direction. This acetylated product was purified by solution in light petroleum (60° to 80°C.), the insoluble portion being a solid was probably a polymerised product. A light golden yellow coloured liquid was obtained from the petroleum fraction corresponding to 66 per cent. of the crude material, the analytical figures being in fair agreement with those required for the monoacetate of the diol (XXIV). This purified monoacetate was used for the following dehydration.

The dehydration was carried by the method used by Isler *et al.*⁴ in the synthesis of vitamin A. A solution of the acetate in light petroleum (100° to 120°C.) after being stabilised with α -tocopherol, was refluxed with iodine for 1 hour under nitrogen. The product obtained was examined spectroscopically. (Fig. 4, curve II.) A new absorption peak produced at 325 $m\mu$, with $E_{1\text{ cm}}^{1\text{ per cent}}$ 245, indicated the formation of 3:7-dimethyl-9-phenylnona-2:4:6:8-tetraene-1-ol acetate (XXV). However, active hydrogen determination gave a figure corresponding to the presence of about 36 per cent of XXIV, which is thought to be responsible for part of the spectrum.

A number of ways are known to achieve this dehydration. In the synthesis of vitamin A ether, Milas *et al.*¹⁷ described the use of *p*-toluenesulphonic acid, pyridine hydrobromide in presence of pyridine, alcoholic potassium hydroxide, phosphorus tribromide or thionyl chloride in conjunction with pyridine, or sodamide in liquid ammonia. The iodine method has the advantage that it catalyses *cis-trans* isomerisation^{22,23}, although it has been reported in the literature^{24,25,26,27} that dehydration usually resulted in a *trans* bonding, semihydrogenation of the triple bond gave in most instances a *cis* double bond²⁸. However, the broadness of the absorption band produced by the product suggests that stereoisomers were probably present.

Saponification of the acetate gave a product containing the free carbinol, 3:7-dimethyl-9-phenylnona-2:4:6:8-tetraene-1-ol (XIII). It showed an absorption maximum at 329 $m\mu$ with $E_{1\text{ cm}}^{1\text{ per cent}}$ 254 (Fig. 4, curve III). Spectroscopically, a benzene ring effects approximately the same bathochromic shift to the absorption peak of a polyene compound as does an extra conjugated double bond. This was found to be the case in α : β -unsaturated ketones by Wilds *et al.*²⁹ Thus, with a ring double bond, methyl substituted, β -ionone has a maximum (at 293 $m\mu$) 4 units towards the longer wave length than benzalacetone (at 289 $m\mu$). 1-Vinylcyclohexene-1 on the other hand, has a maximum (at 230 $m\mu$)³⁰ 10 units towards the shorter wave length than styrene (at 240 $m\mu$)³¹. Hence, it was expected that the maximal absorption of the carbinol (XIII) should be in the neighbourhood of that of vitamin A.

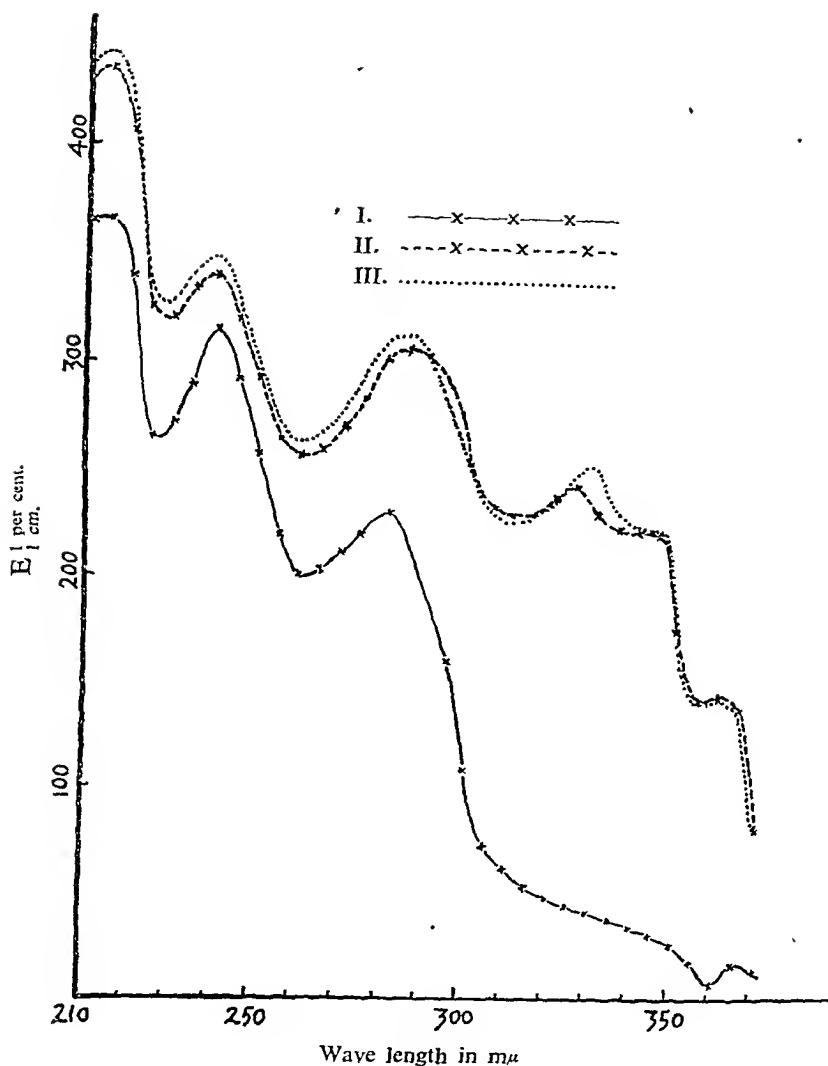


FIG. 4.

It is to be regretted that owing to the great instability of the product obtained, further purification of the sample by chromatographic means was not attainable. The bulk of the sample, stored under nitrogen in the dark, deteriorated during the course of its spectral and biological studies as shown by a loss of the specific absorption properties. The acetate, which was tested biologically, was found to be completely inactive.

Examination of the structure of XIII reveals its differences from vitamin A in two respects: (a) the presence of the benzene ring which brings the whole molecule into coplanarity and hence different from vitamin A slightly in its spatial arrangements. On the other hand, the

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presence of a stable benzene nucleus in the molecule may modify its chemical activity. (b) the absence of the three methyl substituent groups from the ring. They may have a certain specific effect. Therefore it can be concluded that the polyene carbinol side chain alone in the vitamin A molecule is not sufficient, though necessary, for producing vitamin A activity. These results stimulate interest in the synthesis of the corresponding cyclohexenyl analogue of the vitamin (XII) in order to determine whether it possesses any biological activity.

EXPERIMENTAL

All absorption spectroscopic measurements were carried out on solutions in ethyl alcohol. Melting-points are uncorrected.

Benzalacetone. Prepared according to Organic Syntheses³², having m.pt. 42°C.

Ethyl γ -bromocrotonate. Prepared according to the method of Ziegler *et al.*³³. The fraction having b.pt. 91° to 93°C./10mm. Hg. pressure was used.

Reformatski condensation of benzalacetone and ethyl γ -bromocrotonate Benzalacetone (146 g.), ethyl γ -bromocrotonate (193 g.) and benzene (sodium dried, 1000 ml.) were mixed together with zinc wool (washed with acetone and dried, 65.4 g.) and a small crystal of iodine. The whole was heated under a reflux condenser with stirring over a steam bath to start the reaction which occurred a few minutes after the refluxing had started. The heating was then interrupted till the reaction had subsided, the refluxing being then continued for another 4 hours. After cooling the unreacted zinc was collected (12.8 g.). The benzene solution of the complex was decomposed with crushed ice and diluted acetic acid (5 per cent.). Benzene extraction gave 180 g. of a reddish oily liquid after removal of solvent. By subjecting this liquid to distillation at 0.01mm. Hg pressure, partial dehydration occurred at a bath temperature of 40°C. A fraction of benzalacetone, identified by its 2:4-dinitrophenylhydrazone, was recovered at bath temperature 106° to 110°C. (53.7 g.). The distillation was continued at 130°C. for a further half-hour when only a small amount of unidentified material distilled over. The non-distillable material was subjected to high vacuum distillation in a modified short path still of the cold finger type. The material distilled over at 115° to 130°C. (bath temperature) at 10⁻⁴mm. Hg pressure and consisted of a mixture of the hydroxy ester and the dehydrated ester. Active hydrogen (Zer.) corresponding to 0.445 H.

Ethyl-5-methyl-7-phenylhepta-2:4:6-trienoate (crude). The partially dehydrated ester (50 g.) was mixed with half its weight of anhydrous oxalic acid and heated at 110°C. under reduced pressure (10mm. Hg) for 1½ hours. The product was extracted with benzene, the benzene extract being washed with solution of sodium bicarbonate and water. After the removal of benzene, the product was distilled in high vacuum, when the dehydrated ester distilled over at 93° to 98°C. 10⁻⁴ to 10⁻⁵mm. Hg pressure. Weight: 38 g. Active hydrogen (Zer.): negligible. Light absorption:

(Fig. 1, curve I) maximum 340m μ , $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 225; inflection 250 m μ , $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 105. Refractive index: $n_D^{20^\circ\text{C.}}$ ca. 1.55.

5-Methyl-7-phenylhepta-2:4:6-trienoic acid. The dehydrated ester (21 g.) was saponified by shaking with alcoholic potassium hydroxide (7 g. of potassium hydroxide in 77 ml. of alcohol (90 per cent.)) overnight. The soap solution was diluted with water (500 ml.) and extracted with ether which yielded on evaporation only about 0.5 g. of a neutral fraction. The soap solution was then acidified with dilute acetic acid (5 per cent.). The crude acid separated as a gummy precipitate which solidified on standing. By dissolving the crude acid in ether and cooling in a refrigerator overnight, a lemon yellow coloured crystalline acid melting at 190° to 194°C. was obtained. Its colour darkened on standing in air, and it was therefore kept in an atmosphere of nitrogen. Yield: 0.8 g. from about 15 g. of crude acid. Found: C, 77.1; H, 6.56; $\text{C}_{14}\text{H}_{14}\text{O}_2$ requires C, 78.5; H, 6.54 per cent. Active hydrogen (Zer.): 1.05 H. Light absorption (Fig. 1, curve II) Maxima: 342 m μ , $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 1650, log. ϵ 4.548; 249 m μ , $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 418, log. ϵ 3.952. The bulk of the acid was non-crystallisable.

Glycid ester condensation of benzalacetone and ethyl chloroacetate. The general method given by Darzens was followed¹⁰. Benzalacetone (28 g.) and ethyl chloroacetate (freshly distilled, 23.8 g.) were mixed together in a flask and the mixture cooled to -7°C. Alcohol free sodium ethoxide (13 g.) was added in small portions with constant stirring over a period of about 1½ hours. The mixture was stirred at room temperature overnight, then heated over a water bath for 1 hour. After cooling, 100 g. of crushed ice was added followed by the gradual addition of dilute acetic acid (60 ml. of 11 per cent. acid). Extraction with ether and subsequent removal of the solvent gave 43 g. of material which was fractionated. The glycid ester distilled over at 133° to 137°C./0.5 mm. Hg. pressure as a colourless viscous oil. Yield: 10 g. (23 per cent. of theory). It had refractive index: $n_D^{33^\circ\text{C.}}$ 1.5401. Found: C, 72.41; H, 6.74; $\text{C}_{14}\text{H}_{16}\text{O}_3$ requires C, 72.41; H, 6.95 per cent. Saponification value gave its molecular weight as 227: $\text{C}_{14}\text{H}_{16}\text{O}_3$ requires 232.

2-Methyl-4-phenylcrotonaldehyde (XIX). The glycid ester (26.3 g.) in alcohol (40 ml.) was saponified with alcoholic potassium hydroxide (142 ml. of 1.8 N.) by shaking in an atmosphere of nitrogen for 2 hours and then leaving to stand overnight. A small amount of potassium carbonate precipitate formed during this treatment. After dilution with water and acidification, the free glycid acid precipitated as a red coloured gummy solid. This crude acid was not separated and further purified, but the whole mixture was heated over a boiling water-bath. Decarboxylation occurred smoothly and was completed in about 15 minutes when the solid acid changed into an oily liquid. After extraction with ether, the ethereal extract was washed with water and fractionated yielding mainly the crude 2-methyl-4-phenylcrotonaldehyde at 102° to 110°C./0.3 mm. Hg pressure as a pale yellow liquid. Yield, 3 g. (16 per cent. of theory based upon the glycid ester).

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2-Methyl-4-phenylcrotonaldehyde (pure) by improved method. The method used by Isler *et al.*⁴ was followed. Benzalacetone (56 g.) and ethyl chloroacetate (46.5 g.) were mixed in a flask cooled in a solid carbon-dioxide-acetone bath. Sodium ethoxide (26 g.) was added in small portions with stirring as before, the whole being left overnight at room temperature. Alcoholic potassium hydroxide (220 ml. of 1.8 N.) was added to the mixture gradually with constant stirring, cooling as before. After being stirred for 3 hours, the whole was left over the week-end. Water (600 ml.) was then added with cooling followed by extraction with ether. The aqueous saponaceous liquor on acidification gave only a negligible amount of oily liquid, which indicated almost complete decarboxylation of the glycidate under these conditions. The ethereal extract, after being washed with water and dried, was distilled to remove the solvent, giving 53.3 g. of product which was fractionated:

Fraction 1: 80° to 82°C./0.05 mm. Hg pressure, 25 g. $n_D^{20^\circ}$ 1.5539.

Fraction 2: 84° to 92°C./0.05 mm. Hg pressure, 3 g. $n_D^{20^\circ}$ 1.5549.

Fraction 3: 110° to 40°C./0.05 mm. Hg pressure, 3 g. $n_D^{20^\circ}$ 1.5660.

Fraction 1 was apparently the main crop of the required aldehyde which on redistillation at 70°C./0.05 mm. Hg gave a yield of 22 g. of the pure aldehyde (about 40 per cent. of theory overall). The aldehyde gave the following constants: $n_D^{20^\circ}$ 1.5537; $d_4^{20^\circ}$ 1.0105; molecular refraction: found 50.77, calculated for $C_{11}H_{12}O$, 48.94, exaltation 1.83 units. Found: C, 82.11; H, 7.69; $C_{11}H_{12}O$ requires C, 82.50; H, 7.50 per cent. Semicarbazone, leaflets from alcohol, m.pt. 178° to 179°C.; thiosemicarbazone, needles from alcohol, m.pt. 132° to 132.5°C.; 2:4-dinitrophenylhydrazone, red needles from chloroform, m.pt. 188° to 190°C. Found: N, 16.1; $C_{11}H_{12}N_4O_4$ requires N, 16.47 per cent.

Light absorption data (in ethyl alcohol) (Fig. 2).

The aldehyde: Maxima. 229 m μ , $E_1^{1\% \text{ cm}}$ 1021, log ϵ 4.214; 283 to 284 m μ , $E_1^{1\% \text{ cm}}$ 142, log ϵ 3.357.

The semicarbazone: Maximum 266 m μ , $E_1^{1\% \text{ cm}}$ 1581, log ϵ 4.536.

The thiosemicarbazone: Maxima 299 m μ , $E_1^{1\% \text{ cm}}$ 1601, log ϵ 4.409; 249 m μ , $E_1^{1\% \text{ cm}}$ 490, log ϵ 3.895.

Fraction 2 on redistillation yielded mainly the same aldehyde distilling at 70° to 72°C./0.05 mm Hg pressure.

Fraction 3 yielded a 2:4-dinitrophenylhydrazone, but was apparently a complex mixture and was not investigated further.

3-Methylpent-4-en-1-yn-3-ol was prepared by the method given by Heilbron and Jones⁴. The product on careful fractionation through a Widmer column gave the pure carbinol distilling at 66° to 66.5°C./50 mm. Hg pressure. It had refractive index: $n_D^{15^\circ}$ 1.4438 (Lit., $n_D^{15^\circ}$ 1.4490¹⁴; $n_D^{20^\circ}$ 1.4444¹⁵). Active hydrogen: (Zer.) 1.09 H at room temperature, 2.03 H after heating at 100°C.

3-Methylpent-2-en-4-yn-1-ol (XX) was obtained by anionotropic rearrangement of 3-methylpent-4-en-1-yn-3-ol according to Heilbron and

Jones¹⁴. The carbinol distilled at 77° to 78°C./18 mm. Hg pressure. Refractive index $n_D^{17^\circ\text{C.}}$ 1.4850 (Lit., $n_D^{16^\circ\text{C.}}$ 1.4850¹⁴). Active hydrogen (Zer.); 1.93 H. after warming. α -Naphthylurethane: m.pt. 118° to 119°C. (Lit., 119°C.¹⁶).

3:7-Dimethyl-9-phenylnona-2:7-dien-4-yn-1:6-diol (XXII). The condensation was carried out in a similar way to that used by Isler *et al.*⁴ To the Grignard compound of 3-methylpent-2-en-4-yn-1-ol (XXI) (from 5.1 g. of the carbinol) in ether was added a solution of 2-methyl-4-phenylcrotonaldehyde (8.0 g.) in ether (12.5 ml.) over a period of 15 minutes, the whole being cooled in an ice water bath. Vigorous stirring was maintained and a slow stream of nitrogen was passed into the apparatus all the time. After the completion of the addition of the aldehyde, the whole mixture turned into a stiff mass insoluble in ether and was then refluxed over a warm water-bath with efficient stirring when the mass gradually softened. The refluxing was continued for 7 hours. It was then decomposed by shaking with crushed ice and ammonium chloride solution in nitrogen overnight. The product was then extracted with ether, the ethereal extract being dried and fractionated. About 1 ml. of the 3-methylpent-2-en-4-yn-1-ol and a small amount of the 2-methyl-4-phenylcrotonaldehyde were recovered. The product, after being deprived of low-boiling material under 0.04 mm. Hg pressure over a water-bath for 1 hour, was purified by a partition between light petroleum and aqueous methyl alcohol: The product was dissolved in methyl alcohol (100 ml. of 75 per cent.) and extracted with light petroleum (3 quantities, each of 30 ml.). The petroleum fraction which contained some hydrocarbon was not investigated further. The aqueous methyl alcohol fraction was diluted with water (400 ml.) and extracted with ether (4 quantities, each of 70 ml.). After the removal of the last trace of solvent from the ethereal extract at 70° to 80°C./0.05 mm. Hg pressure, 3:7-dimethyl-9-phenylnona-2:7-dien-4-yn-1:6-diol was obtained as a viscous brownish liquid. Yield: 10 g. (78 per cent. of theory). It had the following characteristics: $n_D^{20^\circ\text{C.}}$ 1.5756; $d_4^{19^\circ\text{C.}}$ 1.0673; Molecular refraction: found 79.43, calculated for $\text{C}_{17}\text{H}_{20}\text{O}_2$, F_s 77.22; exaltation: 2.21 units. Found C, 79.86; H, 7.95; $\text{C}_{17}\text{H}_{20}\text{O}_2$ requires C, 79.68; H, 7.88 per cent. Active hydrogen (Zer.): 2.04 H. Light absorption: (Fig. 3, curve I) inflections at 215 $\text{m}\mu$, $E_1^{1\text{ per cent.}}$ about 750; 230 $\text{m}\mu$, $E_1^{1\text{ per cent.}}$ about 615.

Partially poisoned catalyst. A supported catalyst of 5 per cent. palladium over barium sulphate (6 g., prepared according to Organic Syntheses¹⁵) suspended in purified alcohol (30 ml.) was treated with quinoline sulphur poison¹⁶ (0.6 ml.). After being stirred for $\frac{1}{2}$ hour, the catalyst was collected on a filter, washed with a little alcohol and dried *in vacuo*.

3:7-Dimethyl-9-phenylnona-2:4:7-trien-1:6-diol (XXIII). 3:7-Dimethyl-9-phenylnona-2:7-dien-4-yn-1:6-diol (9 g.) was dissolved in methyl alcohol (90 ml.) to which the partially poisoned catalyst (1 g.) was

added. Hydrogenation was carried out under approximately atmospheric pressure. However, after the hydrogenation had proceeded slowly for about 4 hours, the rate of absorption of hydrogen became very slow. Fresh additions of catalyst were made and the hydrogenation stopped when 0.99 mol. proportion of hydrogen was absorbed. Total amount of catalyst used was 2.3 g. The catalyst was removed from the solution and the solvent distilled off in nitrogen, the final trace of volatile matter being removed at 80° to 90°C./0.05 mm. Hg pressure. Yield: 9.0 g. of brownish liquid. Found: C, 80.75; H, 8.84; $C_{17}H_{22}O_2$ requires C, 79.02; H, 8.58 per cent. It had the following characteristics: $n_D^{17^\circ C.}$ 1.5673, $d_4^{17^\circ C.}$ 1.039. Molecular refraction: found: 81.15, calculated for $C_{17}H_{22}O_2$: 78.75; exaltation 1, 2.40 units. Active hydrogen (Zer.): found 0.63 per cent., $C_{17}H_{22}O_2$ requires 0.78 per cent. for 2H. With chloroformic solution of antimony trichloride it gave an instantaneous blue coloration changing rapidly through violet to red. Light absorption: (Fig. 3, curve II) Maxima 215 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 357; 282 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 197.

3:7-Dimethyl-9-phenylnona-2:4:7-trien-1:6-diol monoacetate (XXIV). The diol obtained above (6.5 g.) was dissolved in a mixture of benzene (40 ml.) and dry pyridine (40 ml.). The solution was cooled in a solid carbon dioxide-acetone bath, and freshly distilled acetyl chloride (2.3 g.) was added drop by drop with stirring in nitrogen. After the completion of the addition, the cooling bath was removed and the whole was stirred at room temperature for $\frac{3}{4}$ hour, then refluxed for 1 hour. The mixture was then cooled to below 0°C., decomposed with water and extracted with benzene. Removal of solvent gave the crude monoacetate as a brownish viscous liquid. Yield: 7.4 g. Light absorption: (Fig. 4, curve I) Maxima 215 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 367; 240 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 316; 282 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 232.

Purification of the monoacetate. The crude acetate (6.6 g.) was extracted repeatedly with hot petroleum (100°C.), the residue insoluble in petroleum being a dark brownish gummy solid, probably a polymerised product. The petroleum extract was evaporated to remove the solvent and a light golden yellow liquid* was obtained. Found: C, 75.06; H, 7.63; $C_{19}H_{24}O_2$ requires C, 75.97; H, 8.05 per cent. Refractive index: $n_D^{20^\circ C.}$ 1.557. Active hydrogen (Zer.): 1.1 H.

3:7-Dimethyl-9-phenylnona-2:4:6:8-tetrane-1-ol acetate (XXV). The dehydration was carried out according to Isler *et al.*⁴ The monoacetate obtained from above (4.2 g.), α -tocopherol (40 mg.), were dissolved in light petroleum (80° to 100°C., 70 ml.). Iodine (40 mg.) in light petroleum (10 ml.) was added and the whole was refluxed for 1 hour. After cooling, the solution was washed with solution of sodium thiosulphate and then with water. Removal of solvent gave a dark brown oil. Yield: 3.8 g. With antimony trichloride in chloroform a transient blue colour which changed rapidly through violet to red was produced. Light absorption (Fig. 4, curve II) Maxima 215 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 435; 240 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 340; 282 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 306; 325 m μ , $E_{1\text{ cm}}^{1\text{ per cent.}}$ 245. Active hydrogen: 0.36 H.

3:7-Dimethyl-9-phenylnona-2:4:6:8-tetraene-1-ol (XIII). The acetate (0.5 g.) was dissolved in absolute alcohol (3 ml.), to which, with stirring and cooling, was added alcoholic potassium hydroxide (3 ml. of N) in an atmosphere of nitrogen. After standing overnight, the solution was diluted with water (50 ml.) and extracted with ether. The ethereal extract was washed and dried, and the solvent removed. A brownish viscous oil was obtained, weighing 0.35 g. With antimony trichloride in chloroform: An instantaneous blue coloration which changed rapidly through violet to red. Light absorption: Fig. 4, curve III) Maxima 215 $m\mu$, $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 446; 240 $m\mu$, $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 350; 282 $m\mu$, $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 307; 329 $m\mu$, $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 254.

The authors are indebted to Dr. K. H. Coward and Messrs. Glaxo Laboratories Ltd. for carrying out individual biological tests. C. C. Shen thanks the Directors of Messrs. May and Baker Ltd. for their generous financial support which enabled him to be engaged in the present work.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS CHEMISTRY ANALYTICAL

Benadryl and Pyribenzamine Hydrochlorides, Identification and Differentiation of. T. J. Haley. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 294.) The reactions of 12 precipitants and 16 colorimetric reagents with benadryl hydrochloride (diphenhydramine hydrochloride) and with pyribenzamine hydrochloride (tripelennamine hydrochloride) are described. Only chloroplatinic acid, 5 per cent., could be used by precipitation for identification and differentiation between these two. Benadryl gives a granular orange precipitate of leaf-like crystals in crosses with some cigar-shaped crystals. Pyribenzamine gives a granular orange precipitate of rosettes and sheaves of flat plates on drying. The colour reactions are distinctive; with concentrated sulphuric acid, benadryl gives an orange colour and pyribenzamine a greenish yellow colour; complete destruction of the organic compounds by the strong acid results in a dark brown to black solution unsuitable for qualitative analysis. With potassium dichromate and concentrated sulphuric acid, benadryl forms a yellow solution and pyribenzamine a brown solution. Resorcinol and concentrated sulphuric acid gives an orange and then reddish orange colour with benadryl, which becomes wine-coloured when diluted with water; the same reagent gives a yellowish green and then deep green colour with pyribenzamine which becomes olive-green on dilution with water. Furfural 1 per cent. overlay sulphuric acid gives an orange-brown colour changing to yellow-green on shaking with benadryl, and a black colour which does not alter on shaking with pyribenzamine. Mandelin's reagent gives a red colour with oily red globules with benadryl, and a chocolate-brown colour with pyribenzamine; Marquis' reagent, yields a colour change with benadryl from canary-yellow to reddish-orange to chocolate-brown, and from red to deep reddish-brown with pyribenzamine; Mecke's reagent, gives a canary-yellow then reddish-yellow colour with benadryl, and a nut-brown colour with pyribenzamine; and Fröhde's reagent gives a canary-yellow colour followed by orange and then red with benadryl, but with pyribenzamine yields a pale pink followed by a deep rust colour.

L. H. P.

Progesterone, Photometric Determination of. E. Diding. (*Svensk. Farm. Tidskr.*, 1949, 53, 269.) The method is based on the formation of a red dinitrophenylhydrazone, soluble in chloroform. The colour is stable for at least 24 hours. Details are as follows: 5 ml. of an alcoholic solution, containing 0.25 to 1.25 mg. of progesterone is treated with 3.0 ml. of a freshly-prepared 0.25 per cent solution of 2,4-dinitrophenylhydrazine in 2M hydrochloric acid. The mixture, in a covered beaker, is heated on the water-bath for 15 minutes, then treated with 10 ml. of 2M hydrochloric acid, and heated for a further 30 minutes to remove the alcohol. The precipitate is transferred to a sintered glass filter, washed with hydrochloric acid, and then with water. After drying *in vacuo*, the dinitrophenylhydrazone is dissolved in chloroform to 100 ml., and the extinction is determined at 440 m μ . For solutions in oil, 5 ml. of the solution is dissolved in 10 ml. of hexane and extracted with 4 quantities, each of 5 ml., of alcohol

(90 per cent.). The alcoholic extracts are washed with 20 ml. of hexane, which is then washed with 2 quantities, each of 5 ml., of alcohol. The alcoholic solutions are filtered and evaporated to dryness, and the residue is dissolved in alcohol and treated as before.

G. A

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

nor-Adrenaline in Adrenal Medulla, Evidence for Occurrence

M. Goldenberg, M. Faber, E. J. Alston and E. C. Chargaft. (*Science*, 1949, 109, 534.) By paper chromatography using phenol saturated with water as eluent in an atmosphere of hydrogen chloride it has been found that samples of U.S.P. Reference Standard Epinephrine contained 12 to 18 per cent. of *nor*-adrenaline and that one sample contained as much as 36 per cent. The adrenaline fractions from three chromaffin tissue tumours were found to contain 50 to 90 per cent of *nor*-adrenaline. It is pointed out that adrenaline and *nor*-adrenaline differ significantly in their pharmacological actions both as regards effect on cardiac output and on carbohydrate metabolism. If it is assumed that natural adrenaline as secreted by the adrenal gland maintains a constant content of *nor*-adrenaline, present concepts of adrenal secretion remain valid. If, however, under varying physiological conditions the *nor*-adrenaline content of the secreted natural adrenaline varies then it is considered that current views of the physiology of the adrenal medulla may have to be modified. Under pathological conditions such as in pheochromocytoma, haemodynamic effects and the influence on carbohydrate metabolism are profoundly altered by the high content of *nor*-adrenaline in the secreted medullary hormone. The biological assay of tumour extracts is discussed.

F. H.

BIOCHEMICAL ANALYSIS

Barbiturates in Tissue; Determination by Ultraviolet Absorption Spectrophotometry. G. V. R. Born. (*Biochem. J.*, 1949, 44, 501.)

A procedure based on ultraviolet spectrophotometry is described for the quantitative determination of very small amounts of barbiturates in tissues and blood. The tissue is homogenised and proteins precipitated by ethyl alcohol. The barbiturate in the acidified protein-free filtrate is extracted with ether and passed into alkali. To two samples of this alkaline solution are added phosphate or borate solutions which bring the pH to different known values. The extinctions at 23 m μ of the resulting solutions are measured and from these the concentration of barbiturate in the tissue can be determined by calculation. Complete elimination of contaminants is not attempted. The difficulty due to the simultaneous extraction of barbiturate and impurities absorbing in the same region of the ultraviolet is overcome by the use of differential spectrophotometry, depending on extinction-pH relationships. The procedure is rapid, accurate and sensitive, permitting the extinction of concentrations of barbiturates as low as 1 to 2 μ g./ml. in pure solution.

S. L. W.

Nicotinamide in Biological Materials, Fluorimetric Estimation of. D. K. Chaudhuri and E. Kodicek. (*Biochem. J.*, 1949, 44, 343.) The material under examination (5g.) is cut finely, ground with sand and 0.1N hydrochloric acid (1 to 2 ml.) transferred with 40 ml. of water to a 100-ml. beaker on a boiling water-bath and heated for 30 minutes. After

BIOCHEMICAL ANALYSIS

cooling, hydrochloric acid is added to pH 2, the mixture is centrifuged, the residue washed with 10 ml. of 0.1N hydrochloric acid and again centrifuged. The combined liquids are adjusted to a known volume (usually 40 ml.) with 0.1N hydrochloric acid, 6 ml. of a freshly prepared 25 per cent. solution of metaphosphoric acid is added and the liquid centrifuged after standing for 5 to 10 minutes. The clear solution is adjusted to pH 9.4 to 9.6, heated on a boiling water-bath for 30 minutes, cooled, adjusted to pH 7.2 and made up to 50 ml.; after filtration (Whatman No. 5) it is then treated with arsenogen bromide solution. Three determinations are made—a blank, the unknown filtrate (containing 5 to 25 μ g. of nicotinamide), and the unknown to which is added an internal standard (25 μ g. nicotinamide). After mixing, the three solutions are heated in a water-bath for 4 minutes at 56° to 58°C., cooled and made up to 15 ml. with water. 5N sodium hydroxide (8 ml.) is added, the volume made up to 30 ml. with water and the fluorescence read after 45 minutes at room temperature in the dark. Details of the calculation and of the reproducibility of the results are given; the method estimates the total nicotinamide content, including the free and bound forms. Specific and reproducible results were obtained for biological materials and for cereals, and the results agreed well with the reported micro-biological values. Practically all the vitamin in rat organs and muscles seemed to be present in the form of the amide, bound or free. In bran, no nicotinamide was detected before or after digestion. The breakdown product of the "precursor" of nicotinic acid present in bran appeared to be the free acid and not the amide. Yeast and wheat germ contained about 10 per cent. of the vitamin present in the amide form, bound or free.

R. E. S.

CHEMOTHERAPY

Analgesic Compounds, Potential, Preparation of. D. J. Brown, A. H. Cook and I. M. Heilbron. (*J. chem. Soc.*, 1949, Supp. 1, S106, S111 and S113.) In a search for more potent analgesics than amidone analogues have been prepared and examined. Attempts to introduce thiazolyl groups into 1-diethylamino-3-phenylpentan-4-one were unsuccessful. 4-Methyl-2-(3'-diethylamino-1'-phenylpropyl) thiazole and related compounds were prepared from the corresponding γ -dialkylamino- α -phenylthiobutyramide but it was not found possible to introduce ester or ketone groupings on the *tert*-carbon atom. 5-Carbethoxy-4-methyl-2-(3'-diethylamino-1'-phenylpropyl) thiazole and 4-methyl-2-(4'-diethylamino-2'-acetyl-2'-phenylbutyl)thiazole, obtained as viscous oils, were without significant analgesic action. Introduction of basic groups into 4-phenyl- and 4-methyl-2- α -carbethoxybenzyl thiazole failed to yield analgesic properties. Direct carbethoxylation or propionylation of 2-benzylthiophen followed by introduction of a basic side chain yielded products having analgesic properties. Thus 2-(3'-diethylamino-1'-carbethoxy-1'-phenylpropyl)thiophen had an activity approximately one-third that of pethidine while 2-(3'-morpholino-1'-carbethoxy-1'-phenylpropyl)thiophen appeared to be four times as effective as pethidine. Other compounds prepared, such as 2-phenyl-2-diethylaminoethyl cyclohexanone were inactive.

F. H.

PHARMACOGNOSY

Cascara sagrada, Frangula and Oak Barks, Distinction between the Powders. G. G. du Chatelier. (*Ann. pharm. franc.*, 1948, 6, 507.) The powders of these three barks can be distinguished not only by the

ABSTRACTS

presence or absence of stone cells but by the characters of the crystals in the sheaths surrounding the fibres. *Oak bark*, has prismatic crystals with numerous truncations with poorly defined edges, so that the crystals appear rounded. They are elongated with the long axis at right angles to the fibres. Monoclinic crystals (rhombs) very rare. *Cascara sagrada*: crystals generally square and smaller than those of oak bark. Truncations rare, or if present, sharply defined so that the crystals are more regular in shape than those of oak bark. Monoclinic crystals frequent and mostly square. *Frangula bark*: crystal forms intermediate between those of the other two powders. If the crystals in oak bark are slowly dissolved in hydrochloric acid, a detached lignified envelope can be seen within the pecto-cellulose compartment of the crystal sheath; this detached envelope cannot be seen in the other two barks when treated in like manner.

J. W. F

PHARMACOLOGY AND THERAPEUTICS

Antabuse and Alcohol, Effect of on Respiration and Circulation. E. Asmussen, J. Hald, E. Jacobsen and G. Jorgenson. (*Acta Pharmacol. Toxicol.*, 1948, 4, 297.) Alcohol does not produce circulatory or respiratory symptoms in normal human beings in a dose equivalent to 20 g. of absolute alcohol but when experimental subjects were treated with antabuse 12 hours before the intake of alcohol there was a marked increase in ventilation, a decrease in alveolar carbon dioxide, an increased pulse rate, and a slight increase in cardiac output and oxygen consumption. Antabuse-prepared individuals under the influence of alcohol must form a substance that directly or indirectly increases the irritability of the respiratory centre, and the feeling of dyspnoea is due not to a bronchoconstriction but to this increased irritability. The comparatively slight increase in cardiac output shows that there is no serious risk of too heavy a load on the heart after clinical application of antabuse, even though patients complain of serious palpitation and subjective dyspnoea.

S. L. W

Antabuse and Alcohol, Formation of Acetaldehyde after Ingestion of. J. Hald and E. Jacobsen. (*Acta Pharmacol. Toxicol.*, 1948, 4, 305.) After intake of alcohol, human subjects treated with antabuse show a much higher concentration of acetaldehyde in blood than do untreated individuals. The authors discuss the possibility that the symptoms observed after antabuse treatment may be explained as the result of this increased formation of acetaldehyde.

S. L. W.

Iodophthalein, Excretion from the Human Organism. H. O. Bang and J. Georg. (*Acta Pharmacol. Toxicol.*, 1948, 4, 87.) A quantitative method for the determination of amounts as small as 1 µg. of iodophthalein in faeces and organic fluids is described. Injections of iodophthalein, 500 mg intravenously, were given to normal persons, and the concentrations in plasma and urine determined. After the injection a concentration of 10 to 20 mg. per cent. is obtained. This concentration falls steadily, reaching 2 or 3 mg. per cent. 24 hours after the injection. During the next few days iodophthalein is still demonstrable in the plasma and the concentration reaches zero by about the fifth day. Only small amounts of the substance are excreted in the urine. The great bulk of the substance is excreted in the faeces, the

[Continued on page 992]

LETTERS TO THE EDITOR

The Action of Decamethonium Iodide in Birds

SIR,—The mechanism by which neuromuscular block is produced in mammals varies with different substances. Drugs like *d*-tubocurarine chloride act solely on the motor end plate and render it insensitive to the depolarising action of acetylcholine. On the other hand decamethonium iodide can set up propagated contractions when applied sufficiently suddenly (Zaimis¹) and, in any case, causes a depolarisation which extends to the motor end plate as well as to the muscle fibre (Paton and Vianna Dias²); it therefore produces neuromuscular block. Superficially, the paralysis produced in mammals by such an action is indistinguishable from that produced by the curarines. The position however is different when animals are used in which depolarisation of a muscle fibre produces not only electrical inexcitability but, in addition, contracture. This is the case with amphibian and avian muscle, as also with mammalian muscle after denervation. The contracture produced by decamethonium iodide on amphibian and denervated mammalian muscle has been described elsewhere (Paton and Zaimis³, Zaimis¹). The present experiments deal with avian muscle.

In adult fowls or chicks, and in pigeons, an intravenous injection of decamethonium iodide was found to cause a rigid extension of the limbs and retraction of the head (see Figure). If the dose is lethal the animal dies in this rigid condition, if the dose is below the lethal level the recovery is abrupt. Decamethonium iodide is highly active, 0.05 mg/kg. causing spasticity for about 3 minutes in chicks. This is a peripheral effect and the shortening of the muscle is probably a true contracture like that described by Langley as produced in the fowl by nicotine. The excitability of the muscle to nerve stimulation was found to be reduced. The full proof naturally requires electrical analysis of the condition. This reaction of avian muscle to decamethonium iodide is a further confirmation that the action is essentially like that of acetylcholine (Zaimis¹). Tubocurarine chloride on the other hand causes the usual paralysis in birds (see Figure): a dose of 0.5 mg./kg. producing a paralysis that lasts for about 10 to 20 minutes in chicks. Succinylcholine dibromide, another synthetic substance causing neuromuscular block (Bovet *et al.*⁴, Walker⁵), and tetramethylammonium iodide which have both been found to depolarise muscle fibres in a similar way to decamethonium iodide (Paton and Vianna Dias²) produce the same spastic conditions when injected intravenously in birds. On the other hand tri-(diethylamino-ethoxy)-benzene triethyl iodide (Bovet *et al.*⁶), now called flaxedil, a synthetic substance, curare-like in most of its actions, causes in birds a paralysis like that of *d*-tubocurarine. From these findings it appears that not only amphibian muscle and denervated mammalian muscle but also avian muscle may be used as a test in differentiating a true "curare-like" blocking action from the superficially similar resultant action of substances like decamethonium iodide. The advantage of using the test on avian muscle is the ease with which the difference in the action of these two groups of drugs can be strikingly illustrated.

LETTERS TO THE EDITOR

We are grateful to Dr. J. Walker for the supply of succinoylcholine dibromide. This work is being done with the aid of a grant from the Medical Research Council.

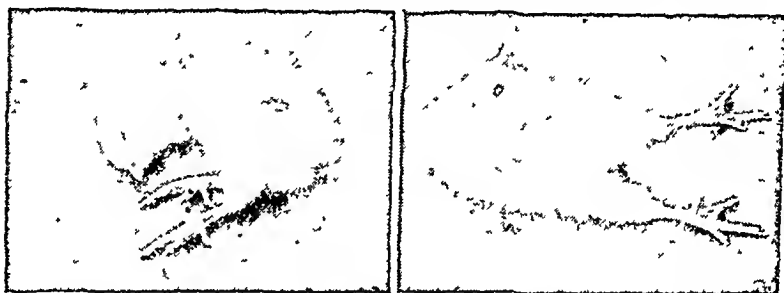
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November 1, 1949.

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A comparison of the effects of an intravenous injection of decamethonium iodide (right) and *d*-tubocurarine chloride (left)

ABSTRACTS (continued from page 990)

excretion usually continuing for 8 to 10 days after the injection. A quantitative balance test was made on two persons and in both about 75 per cent. of the amount injected was recovered. The authors suggest that the balance is excreted in very small amounts (less than 1 mg. daily) over a period of several months after the injection.

S. L. W.

Methyl Alcohol and Formic Acid Excretion in Man. A. LUND. (*Acta Pharmacol. Toxicol.*, 1948, 4, 205.) After intake of small quantities of methyl alcohol (10 to 20 ml.) by human subjects no methyl alcohol was found in the blood in the course of 48 hours, and the concentration of formic acid in the urine was normal (6.5 to 12.8 mg. within 24 hours). Following intake of large amounts of methyl alcohol (50 ml.) this substance could be demonstrated in the blood (25 to 120 mg. per cent.) after 48 hours: formic acid could also be demonstrated in the blood (2.6 to 7.6 mg. per cent.) and in increased amounts in the urine (up to 2050 mg. per cent. within 24 hours). This increased excretion of formic acid after large methyl alcohol intake reaches its maximum in from 1 to 3 days and is characteristic of methyl alcohol poisoning: cases of poisoning with methyl chloride do not show this increase. The author describes 5 fatal cases of methyl alcohol poisoning and gives figures of methyl alcohol and formic acid concentrations in blood and urine.

S. L. W.

